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PATENT
016303-0024300

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Jeffery J. Wheeler *et al.*

Application No.: 09/431,594

Filed: November 1, 1999

For: LIPID-NUCLEIC ACID
PARTICLES PREPARED VIA
HYDROPHOBIC LIPID-NUCLEIC
ACID COMPLEX INTERMEDIATE
AND USE FOR GENE TRANSFER

Customer No.: 20350

Confirmation No. 8936

Examiner: Jane J. Zara

Art Unit: 1635

**Declaration of Ian MacLachlan, Ph.D.
Under 37 C.F.R. § 1.132**

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Ian MacLachlan, Ph.D., state and declare as follows:

1. All statements herein made of my own knowledge are true, and statements made on information or belief are believed to be true and correct.
2. I hold a Ph.D. (1994) from the University of Alberta, and a Bachelor of Science degree (1988) from the University of Alberta. I am presently the Chief Scientific Officer for Protiva Biotherapeutics, Inc. (Burnaby, Canada).
3. My field of expertise is nucleic acid delivery and molecular gene therapy. I have authored twenty-five publications in the field of nucleic acid delivery technology, molecular gene therapy and molecular genetics, and I am a member of the American Society of Gene Therapy and the Science Council of British Columbia, Health Technology Committee.
4. Attached hereto as Exhibit A is a true copy of my *curriculum vitae* and a list of publications of which I am an author or co-author.

5. I have read and am familiar with the contents of the above-referenced patent application. In addition, I have read the Final Office Action, mailed September 30, 2004, received from the United States Patent & Trademark Office in the above-referenced patent application. It is my understanding that the Examiner is concerned that claims 42, 44-61 and 63-75 are anticipated under 35 U.S.C. § 102(e) over U.S. Patent No. 5,820,873 ("Choi *et al.*"), and that claims 42, 44-61, 63-64 and 67-75 are anticipated under 35 U.S.C. § 102(e) over U.S. Patent No. 5,885,613 ("Holland"). For the reasons set forth herein, the Examiner's concerns are overcome.

6. This declaration is provided to clarify the distinguishing elements of the presently claimed invention and to demonstrate that the methods described in Choi *et al.* and Holland *et al.* cannot be used to produce the presently claimed nucleic acid-lipid particles.

7. The presently claimed invention is directed, *inter alia*, to nucleic acid-lipid particles for introducing a nucleic acid into a cell either *in vitro* or *in vivo*. More particularly, independent claim 42 reads as follows:

42. A nucleic acid-lipid particle for introducing a nucleic acid into a cell, said particle comprising a cationic lipid, a conjugated lipid that inhibits aggregation of particles, and a nucleic acid, wherein said nucleic acid is encapsulated in the lipid of said particle and is resistant in aqueous solution to degradation with a nuclease.

8. The specification teaches methods of making lipid-nucleic acid particles via novel, hydrophobic nucleic acid-lipid intermediate complexes. Manipulation of these complexes in either detergent-based or organic solvent-based systems leads to nucleic acid-lipid particles, wherein the nucleic acid in the nucleic acid-lipid particles is protected from nuclease degradation.

9. It is my understanding that Choi *et al.* and Holland *et al.* are cited by the Examiner as allegedly disclosing nucleic acid-lipid particles that meet the structural limitations of the particles produced by the methods of the instant invention (*see*, pages 2 and 5 of the Office Action, respectively).

10. I have reviewed the Choi *et al.* and Holland *et al.* patents, and it is my opinion that *neither* Choi *et al.* nor Holland *et al.* teach (or even suggest) a nucleic acid-lipid particle, wherein the nucleic acid in the nucleic acid-lipid particle is encapsulated in the lipid portion of the particle and is

resistant in aqueous solution to degradation with a nuclease as is recited in claim 42 and, in turn, dependent claims 44-75.

11. A perusal of Choi *et al.* reveals that the Examples set forth methods for loading therapeutic agents, *e.g.*, vincristine, into liposomes. More particularly, Example 9 sets forth the following loading (or encapsulation) method:

The dry lipid was hydrated with 300 mM citrate buffer, pH 4.0. Following extrusion, the vesicles (100 mg/mL) were added to a solution of vincristine (Oncovin; 1 mg/ml) to achieve a drug:lipid ration of 0.1:1. The exterior pH of the liposome/vincristine mixture was raised to pH 7.0-7.2 by titration with 500 mM sodium phosphate and immediately the sample was heated to 60°C for 10 minutes to achieve encapsulation of the vincristine.

See, Example 9, column 21, lines 11-18. Example 10 sets forth a similar loading/encapsulation procedure for loading vincristine into liposomes (see, Example 10, column 21, line 62 through column 22, line 9).

12. The loading/encapsulation methods disclosed in Choi *et al.* are useful for loading small molecules (*e.g.*, vinca alkaloids, *etc.*) into liposomes, but are not useful for loading nucleic acids (*e.g.*, oligonucleotides, plasmid DNA, *etc.*) into liposomes because nucleic acids do *not* readily cross intact lipid membranes. As such, if one were to use the loading/encapsulation methods disclosed in Choi *et al.* and were to add external plasmid DNA to preformed liposomes in aqueous buffer, one would not expect to see any entrapment of the plasmid DNA in the liposomes. Again, this is because nucleic acids do not readily cross intact lipid membranes.

13. Thus, when Choi *et al.* state that cationic carriers of DNA can be improved through the addition of PEG lipids, such as the PEG-ceramide conjugates disclosed and claimed therein, Choi *et al.* are referring to the preformed cationic liposome carriers that are then complexed with DNA to form lipoplexes (*i.e.*, nucleic acid-cationic liposome complexes). In fact, the examples provided in Choi *et al.* that are directed to such preformed cationic liposomes demonstrate that aggregation of the cationic liposomes alone (no DNA) in the presence of serum (most serum proteins carry a net negative charge) can be inhibited if the liposomes contain a PEG-ceramide conjugate. Thus, the methods of Choi *et al.* are directed to forming nucleic acid-cationic liposome complexes, which are structurally and

functionally different from the nucleic acid lipid particles made using the methods of the present invention, *i.e.*, nucleic acid-lipid particles wherein a nucleic acid component is encapsulated in the lipid component and is resistant in aqueous solution to degradation to nuclease.

14. A perusal of Holland *et al.* reveals that the teachings provided therein directed to the delivery of nucleic acids are essentially the same as those provided in Choi *et al.* More particularly, Holland *et al.* states:

For the delivery of therapeutic agents, the fusogenic liposomes of the present invention can be loaded with a therapeutic agent and administered to the subject requiring treatment. The therapeutic agents which can be administered using the fusogenic liposomes of the present invention can be any of a variety of drugs, peptides, proteins, DNA, RNA or other bioactive molecules. Moreover, cationic lipids may be used in the delivery of therapeutic genes or oligonucleotides intended to induce or to block production of some protein within the cell. *Nucleic acid is negatively charged and must be combined with a positively charged entity to form a complex suitable for formulation and cellular delivery.*

Cationic lipids have been used in the transfection of cells in vitro and in vivo. . . . The efficiency of this transfection has often been less than desired, for various reasons. *One is the tendency for cationic lipids complexed to nucleic acid to form unsatisfactory carriers. These carriers are improved by the inclusion of PEG lipids.*

See, column 12, lines 14-26 of Holland et al. (emphasis added).

Clearly, as with Choi *et al.*, the teachings of Holland *et al.* are directed to forming nucleic acid-cationic liposome complexes, which are structurally and functionally different from the presently claimed nucleic acid-lipid particles, wherein the nucleic acid component is encapsulated in the lipid component and is resistant in aqueous solution to degradation with a nuclease.

15. As of the filing date of the Choi *et al.* and Holland *et al.* patents *i.e.*, 1994-1995, the state-of-the-art was to prepare cationic liposomes and, then, to complex the preformed cationic liposomes with DNA in an aqueous solution to form DNA-cationic liposome complexes (*i.e.*, lipoplexes). Given that DNA does not readily cross lipid membranes and that the cationic lipids present in the external membrane of the vesicles would electrostatically interact with the negatively charged DNA, the mixing of DNA with preformed cationic liposomes in aqueous solution does *not* result in

entrapment of DNA within the internal, aqueous space of the liposomes. Moreover, the lipoplexes formed by the methods of Choi *et al.* and Holland *et al.* are ill-defined, are only partially protected from nucleases, are heterogeneous in size and are rapidly cleared from the circulation (*see*, Figure 2 of Wheeler *et al.*, *Gene Therapy*, 6:271-281 (1999); and Figure 1 of Monck *et al.*, *J. Drug Targeting*, 7(6):439-452 (2000), copies of which are attached hereto as Exhibits B and C).

16. In contrast to the teachings of Choi *et al.* and Holland *et al.*, the present invention provides novel methods by which nucleic acids (*e.g.*, oligonucleotides, plasmid DNA, *etc.*) are entrapped, *i.e.*, encapsulated, within individual cationic liposomes that include a conjugated lipid, such as a PEG-lipid conjugate. As explained in the specification and as set forth in the presently pending claims, the PEG-lipid conjugate prevents aggregation of the particles during formation, thereby resulting in nucleic acid-lipid particles of a homogeneous and defined size containing nucleic acid that is fully encapsulated in the lipid bilayer such that the nucleic acid is completely protected from nuclease degradation. This is in stark contrast to the lipoplexes that would be formed based on the cationic liposomes of Choi *et al.*

17. To further demonstrate that the loading/encapsulation methods of Choi *et al.* and Holland *et al.* do *not* produce the presently claimed nucleic acid-lipid particles, a series of experiments using the methods of Choi *et al.* and Holland *et al.* were conducted under my supervision. A lipid solution containing a total of 2.22 μ moles lipid and comprising DOPE:DODAC:PEG-ceramide C14 (82.5:7.5:10 molar percent), was prepared by dissolving these lipids in chloroform. Nitrogen gas was used to drive off chloroform to form a lipid film. The lipid film was then hydrated with 2 ml phosphate buffered saline (pH 7.4) containing 50 or 100 μ g of nucleic acid (*i.e.*, plasmid DNA) to generate liposomal samples with drug (*i.e.*, nucleic acid):lipid ratios of 22.5 and 45 μ g/ μ mol. The resulting suspension was subjected to 5 rounds of freezing in liquid nitrogen and thawing in a 37°C water bath, to increase homogeneity of the resulting multilamellar vesicles. These samples were then extruded 10 times through 2 stacked 100 nm polycarbonate filters using a 10-mL Extruder (Northern Lipids Inc.) and nitrogen gas at 400-600 psi. Nucleic acid encapsulation was determined using membrane-impermeable Picogreen which fluoresces in the presence of plasmid DNA. The proportion of nucleic acid encapsulated in the liposomes was determined by measuring the fluorescence intensity of the Picogreen before and after the addition of the detergent Triton X-100.

Plasmid encapsulation was extremely inefficient at both of the nucleic acid:lipid ratios (*i.e.*, as low as 7% and at best only 15%) examined (*see*, Exhibit D). Moreover, at least 98% of the plasmid DNA was lost on the extrusion filters (*see*, Exhibit D). Particle sizes for all of these extruded samples were all considerably larger than 100 nm. These results unequivocally demonstrate that the loading/encapsulation methods described in Choi *et al.* and Holland *et al.* do *not* produce liposomes that encapsulate plasmid DNA, *i.e.*, nucleic acid-lipid particles resistant in aqueous solution to degradation with a nuclease.

18. In view of the foregoing, it is my opinion that *neither* the Choi *et al.* patent nor the Holland *et al.* patent teach (or even suggest) the nucleic acid-lipid particles recited in claims 42 and 44-75 because *neither* Choi *et al.* nor Holland *et al.* teach (or even suggest) (1) nucleic acid-lipid particles, wherein the nucleic acid in the nucleic acid-lipid particles is encapsulated in the lipid component of the particle and is resistant in aqueous solution to degradation with a nuclease, or (2) methods for making such nucleic acid-lipid particles. Moreover, it has been unequivocally demonstrated that the loading/encapsulation methods described in Choi *et al.* and Holland *et al.* do not lead to the presently claimed nucleic acid-lipid particles resistant in aqueous solution to degradation with a nuclease.

19. I further declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that any such willful false statements may jeopardize the validity of the application or any patent issuing thereon.



Dated: March 30, 2005

Ian MacLachlan, Ph.D.

CURRICULUM VITAE
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BIOGRAPHIC DATA

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EDUCATION

May 1988 - Jun 1994 **Ph.D. (Biochemistry)**
University of Alberta, Edmonton, Canada,
& Department of Molecular Genetics, University of Vienna, Austria.
Sep 1985 - May 1988 **B.Sc. (Biochemistry)**
University of Alberta, Edmonton, Canada.
Sep 1982 - May 1984 **Biological Sciences**
University of Calgary, Calgary, Canada.

EXPERIENCE

Sep 2000 - Present **Chief Scientific Officer**
Protiva Biotherapeutics, Inc.,
Burnaby, BC, Canada. Development of Non-Viral Nucleic Acid Delivery Systems
for Cancer, Inflammatory and Infectious Disease.

Jul 1996 - Aug 2000 **Team Leader / Research Scientist**
Inex Pharmaceuticals Corporation
Burnaby, BC, Canada. Non-Viral Cancer Gene Therapy.
Suicide Gene Therapy, Pharmacology, Vector Development,
Tumor Modeling, Inducible Gene Expression.

Jul 1994 - Jun 1996 **Research Fellow**
Howard Hughes Medical Institute
Department of Internal Medicine
University of Michigan, USA.
Supervisor: Dr. G.J. Nabel TNF Mediated Activation of NF- κ B and the HIV LTR
Adenoviral Gene Therapy for Restenosis.
The Role of NF- κ B in Vertebrate Development.

May 1988 - Jun 1994 **Graduate Student**
Lipid and Lipoprotein Research Group
University of Alberta, AB, Canada.
& Dept. of Molecular Genetics
University of Vienna, Austria.
Supervisor: Dr. Wolfgang Schneider Molecular Genetics of the Lipoprotein Receptor Family.
Characterization of the Receptor Mediated Uptake of
Riboflavin Binding Protein Including Cloning and
Characterization of the rd Mutant.

Jan 1988 - Apr 1988 **Undergraduate Research**
University of Alberta, AB, Canada.
Supervisor: Dr. Wayne Anderson Computerized Sequence Analysis of Lipoproteins,
Crystallography of Membrane Proteins.

Sep 1987 - Dec 1987	Undergraduate Research University of Alberta, AB, Canada. Supervisor: Dr. Wolfgang Schneider	Purification and Characterization of Apolipoprotein VLDL-II, an Inhibitor of Lipoprotein Lipase.
Summer 1987	Undergraduate Research Bamfield Marine Station, Canada. Supervisor: Dr. Ron Ydenberg	Behavioral Analysis of the Polychaete, <i>Eudistilia vancouveri</i> .
May 1983 - Dec 1986	Programmer Canadian Hunter Exploration Ltd. Calgary, Alberta, AB, Canada.	Programming of Oil and Gas Reservoir Simulations and Data Analysis Tools Used to Guide the Exploration Efforts of an Oil and Gas Company.

TRAINING

June 2004	American Society of Gene Therapy/ USFDA	Long Term Follow-up of Participants in Human Gene Transfer Research
March 2003	American Society of Gene Therapy / USFDA	Non-Clinical Toxicology in Support of Licensure of Gene Therapies
Sept 2002	Protiva Biotherapeutics	WHMIS and Chemical Safety Retraining
Sept 2002	TLM Consulting	Basic GMP Training
June 2002	American Society of Gene Therapy / USFDA	Clinical Gene Transfer Comprehensive Review Course
Apr 2002	TLM Consulting	Introduction to Gene Therapy Clinical Trials and GLP/GMP
Jul 2001	Protiva Biotherapeutics	Cytotoxic Drug Training
May 2001	American Society of Gene Therapy / USFDA	Clinical Gene Transfer Training Course
Jun – Sep 1998	Leadership Edge Consulting	Lab-to-Leader Training Program Project Management, Coaching, Team Management
Oct 1997	Pape Management Consulting	Project Management Training II
May 1997	University of British Columbia	Radionuclide Safety and Methodology
Feb 1997	Pape Management Consulting	Project Management Training I

AWARDS AND DISTINCTIONS

1995 - 1998	Medical Research Council of Canada Fellowship
1993	Mary Louise Imrie Graduate Award, Faculty of Graduate Studies and Research, University of Alberta
1992 - 1994	Austrian Fonds zur Förderung der Wissenschaftlichen Forschung (Austrian Ministry of Science Scholarship)
1989 - 1993	Heart and Stroke Foundation of Canada Research Trainee
1982	Rutherford Scholarship

AFFILIATIONS AND MEMBERSHIPS

- 1999 - 2002 Science Council of British Columbia - Health Technology Committee
1998 - Present American Society of Gene Therapy, Member
2004 - Present American Society of Gene Therapy - Non-viral Vectors Committee

PUBLICATIONS

Judge, A.D., Sood, V., Shaw, J.R., Fang, D., McClintock, K. and MacLachlan, I., *Synthetic siRNA Stimulate the Mammalian Innate Immune Response in a Sequence Dependent Manner*, In Press: Nature Biotech, 2005.

Heyes, J., Palmer, L.R., Bremner, K. and MacLachlan, I., *Cationic Lipid Saturation Influences Intracellular Delivery of Encapsulated Nucleic Acids*, In Press: Journal of Controlled Release, 2005.

Ambegia, E.G., Ansell, S., Cullis, P.R., Heyes, J.A., Palmer, L.R. and MacLachlan, I., *Stabilized Plasmid-Lipid Particles Containing PEG-Diacylglycerols Exhibit Extended Circulation Lifetimes And Tumor Selective Gene Expression*, In Press: Biochim Biophys Acta, 2005.

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MacLachlan, I., Cullis, P.R., Graham, R.W., Synthetic Virus Systems for Systemic Gene Therapy. In: *Gene Therapy: Therapeutic Mechanisms and Strategies*, Smyth-Templeton, N., Lasic, D.D., (Eds.) Marcel Dekker, New York, 2000.

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SELECTED ABSTRACTS

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Finn, J.D., Lee, A., MacLachlan, I., Cullis, P.R. *The Development and Characterization of a Cytoplasmic Expression System Based on the T7 Phage RNA Polymerase Protein*. American Society of Gene Therapy, 5th Annual Meeting, June 5-9, 2002.

Sandhu, A., Verheul, R., de Jong, S., MacLachlan, I., Cullis, P. *Enhancing the Intracellular Delivery Characteristics of Stable Plasmid-Lipid Particles*. American Society of Gene Therapy, 5th Annual Meeting, June 5-9, 2002.

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Kyla, C., Cullis, P., Carr, K., Murray, M., Shaw, J., Palmer, L., MacLachlan, I. *Effect of Cationic Lipid Structure on the Pharmacology and Resulting Transfection Activity of Stabilized Plasmid Lipid Particles (SPLP)*. American Society of Gene Therapy, 4th Annual Meeting, May 30-June 3 2001.

Wong, T., Wong, K., Cullis, P., Fenske, D., MacLachlan, I., Sandu, A., Lo, E. *Optimizing the Transfection Potency of Stable Plasmid-Lipid Particles Based on the Endosomal Release Parameter*. American Society of Gene Therapy, 4th Annual Meeting, May 30-June 3 2001.

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Ansell, S., Currie, K., Ambegia, E., Cullis, P., Carr, K., MacLachlan, I., Murray, M. *Stabilized Plasmid Lipid Particles Containing Diacylglycerol Anchored PEG Lipids: In vitro and In Vivo Characterization*. American Society of Gene Therapy, 4th Annual Meeting, May 30-June 3 2001.

Ambegia, E., Cullis, P., Fenske, D., Palmer, L., MacLachlan, Ian., Murray, M. *Programmable Pharmacokinetics, Disease Site Targeting and Tissue Specific Gene Expression of Stable Plasmid-Lipid Particles for Systemic Gene Delivery and Expression*. American Society of Gene Therapy, 4th Annual Meeting, May 30-June 3 2001.

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MacLachlan, I., Fenske, D., Palmer, L., Wong, K., Lam, A., Chen, T., Cullis, P. *Elimination of PEG-Lipid mediated Inhibition of Transfection*. Third Annual Meeting of the American Society of Gene Therapy, May 31-June 4, 2000.

Ahkong, L., Airess, R., Harasym, T. Hope, M., Klimuk, S., Leng, E., MacLachlan, I., Semple, S.C., Tam, P. and Cullis, P.R., *Pre-clinical Studies with Liposomal Mitoxantrone: Formulation, Pharmacokinetics, Toxicity and Efficacy*, 7th Liposome Research Days, April 12-15, 2000.

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Graham, R.W., Tam, P., Lee, D., Thompson, J., Giesbrecht, C., Lee, A., Thompson, V., MacLachlan, I. *A Gene Specific Increase in the Survival of Tumor Bearing Mice Following Systemic Non-Viral Gene Therapy*. American Society of Gene Therapy, 2nd Annual Meeting, June 9-13, 1999.

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Buchkowsky, S., Ayres, S., Graham, R., MacLachlan, I. *Liposomal Encapsulation of Ganciclovir Results in Improved Pharmacokinetics and Biodistribution*. American Society of Gene Therapy, 1st Annual Meeting, May 28-31st, 1998.



Stabilized plasmid-lipid particles: construction and characterization

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A detergent dialysis procedure is described which allows encapsulation of plasmid DNA within a lipid envelope, where the resulting particle is stabilized in aqueous media by the presence of a poly(ethyleneglycol) (PEG) coating. These 'stabilized plasmid-lipid particles' (SPLP) exhibit an average size of 70 nm in diameter, contain one plasmid per particle and fully protect the encapsulated plasmid from digestion by serum nucleases and *E. coli* DNase I. Encapsulation is a sensitive function of cationic lipid content, with maximum entrapment observed at dioleyldimethylammonium chloride (DODAC) contents of 5 to 10 mol%. The formulation process results in plasmid-trapping efficiencies

of up to 70% and permits inclusion of 'fusogenic' lipids such as dioleoylphosphatidylethanolamine (DOPE). The *in vitro* transfection capabilities of SPLP are demonstrated to be strongly dependent on the length of the acyl chain contained in the ceramide group used to anchor the PEG polymer to the surface of the SPLP. Shorter acyl chain lengths result in a PEG coating which can dissociate from the SPLP surface, transforming the SPLP from a stable particle to a transfection-competent entity. It is suggested that SPLP may have utility as systemic gene delivery systems for gene therapy protocols.

Keywords: plasmid encapsulation; nonviral gene delivery; intracellular delivery; gene therapy; liposomes

Introduction

Currently available gene delivery systems for gene therapy protocols have limited utility for systemic applications. Viral systems, for example, are rapidly cleared from the circulation, limiting potential transfection sites to 'first-pass' organs such as the lungs, liver and spleen. In addition, these systems induce immune responses which compromise transfection resulting from subsequent injections. In the case of nonviral systems such as plasmid DNA-cationic lipid complexes (lipoplexes), the large size and positively charged character of these aggregates also result in rapid clearance, and the highest expression levels are again observed in first-pass organs, particularly the lungs.^{1–4} Plasmid DNA-cationic lipid complexes can also result in toxic side-effects both *in vitro*⁵ and *in vivo*.⁶

The need for a gene delivery system for treatment of systemic disease is obvious. For example, for cancer gene therapy there is a vital need to access metastatic disease sites, as well as primary tumors. Similar considerations apply to other systemic disorders, such as inflammatory diseases. The design features for lipid-based delivery systems that preferentially access such disease sites are increasingly clear. It is now generally recognized that preferential delivery of anticancer drugs to tumor sites

following intravenous injection can be achieved by encapsulation of these drugs in large unilamellar vesicles (LUVs) exhibiting a small size (<100 nm diameter) and extended circulation lifetimes (circulation half-life in mice >5 h).^{7–9} The accumulation of these drug delivery systems at disease sites, which includes sites of infection and inflammation as well as tumors, has been attributed to enhanced permeability of the local vasculature in diseased tissue.¹⁰

A gene delivery system containing an encapsulated plasmid for systemic applications should therefore be small (<100 nm diameter) and must exhibit extended circulation life-times to achieve enhanced delivery to disease sites. This requires a highly stable, serum-resistant plasmid-containing particle that does not interact with cells and other components of the vascular compartment. In order to maximize transfection after arrival at a disease site, however, the particle should interact readily with cells at the site, and should have the ability to destabilize cell membranes to promote intracellular delivery of the plasmid. In this work, we show that a straightforward detergent dialysis procedure can produce stabilized plasmid-lipid particles (SPLP) which satisfy the demands of plasmid encapsulation, small size and serum stability. Furthermore, we show that the transfection properties of these systems can be modulated by employing poly(ethyleneglycol) (PEG) coatings which can dissociate from the SPLP, transforming the particle from a stable particle to a transfection-competent entity.

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Results

Entrapment of plasmid DNA into lipid particles by employing detergent dialysis

Previous work has shown that incubation of plasmid DNA with cationic lipids can result in a hydrophobic particle which is soluble in organic solvent.¹¹ It is of interest to determine whether this hydrophobic particle can be surrounded by an outer monolayer of lipid, which would then result in small, plasmid-containing particles stabilized in an aqueous medium. Detergent dialysis is a logical technique for achieving this, as the detergent may be expected to solubilize the hydrophobic plasmid DNA-cationic lipid particles. The addition of phospholipid and subsequent removal of detergent by dialysis could then result in the exchange of the solubilizing detergent with phospholipid, leaving particles which are stable in aqueous suspension.

Initial experiments employed the cationic lipid DODAC, the plasmid pCMVCAT, the non-ionic detergent octylglucopyranoside (OGP) and the bilayer-forming lipid palmitoyloleoylphosphatidylcholine (POPC). When DODAC was added to plasmid in distilled water, the formation of large (>1000 nm diameter) precipitates was observed. However, the subsequent addition of OGP (200 mM) resulted in solubilization of the precipitate, forming an optically clear suspension consistent with entrapment of hydrophobic plasmid DNA-cationic lipid particles within detergent micelles. This optically clear quality was maintained when POPC solubilized in OGP was added. However, during dialysis to substitute the detergent associated with the particles for POPC, extensive precipitation of the suspension was observed. A method to stabilize the plasmid-containing particles against aggregation and precipitation during the dialysis process was therefore required.

Previous studies have shown that a PEG coating can prevent aggregation of LUVs induced by covalent coupling of protein to the surface of the LUVs,¹² and can inhibit fusion between LUVs.¹³ It was therefore of interest to determine whether the stabilizing properties of a PEG coating could prevent aggregation during dialysis. However, the use of the standard PEG-phosphatidylethanolamine (PEG-PE) was contraindicated because the PEG-PE molecule bears a net negative charge and could displace the cationic lipid from the plasmid, as has been noted for other negatively charged lipids.¹⁴ As a result, PEG₂₀₀₀ was linked to ceramide as the hydrophobic anchor to produce a neutral molecule. Two ceramide anchors were synthesized which differed in the length of the ceramide acyl chain (CerC₁₄ and CerC₂₀). When 10 mol% PEG-CerC₂₀ was incorporated in the detergent mixture with POPC, DODAC and plasmid DNA, precipitation was not observed during detergent dialysis. Further, a proportion of the plasmid was encapsulated, as measured by recovery of DNA after elution on a DEAE-Sepharose CL-6B anion exchange column. As shown in Figure 1a, the encapsulation achieved is a sensitive function of the DODAC content, with encapsulation levels of 30% or higher at about 9% to 12% DODAC. It should be noted that addition of plasmid to preformed vesicles with the same lipid composition, followed by DEAE chromatography, resulted in complete plasmid retention on the column.

These results suggest that SPLP can be produced by

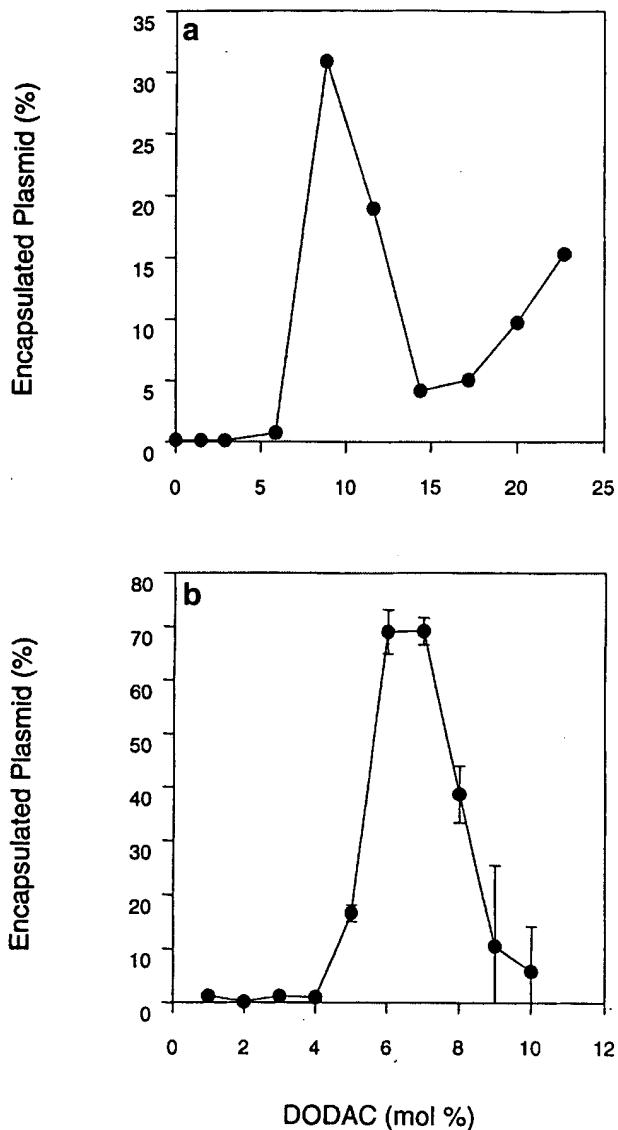


Figure 1 Effect of DODAC concentration on the encapsulation efficiency of plasmid DNA (pCMVCAT) in SPLP. (a) Lipid composition POPC, DODAC and 10 mol% PEG-CerC₂₀. (b) Lipid composition DOPE, DODAC and 10 mol% PEG-CerC₂₀. Lipid (10 mg/ml total), dissolved in octylglucoside (0.2 M), was mixed with plasmid DNA (50 µg/ml) in a total volume of 1 ml to form an optically clear solution. This was then placed in a dialysis tube (12–14 000 molecular weight cutoff) and dialyzed against HBS for 36 h at 20°C. Encapsulation efficiency was determined following removal of unencapsulated plasmid by anion exchange chromatography, as outlined in Materials and methods.

detergent dialysis employing a POPC/DODAC/PEG-CerC₂₀ (79:11:10; mol:mol:mol) lipid mixture. However, it has been shown that when POPC is employed as a 'helper' lipid in plasmid DNA-cationic lipid complexes, very low transfection rates are observed, whereas when dioleoylphosphatidylethanolamine (DOPE) is present, much higher transfection rates are achieved.¹⁵ The encapsulation properties of DOPE/DODAC/PEG-CerC₂₀ lipid mixtures were therefore investigated. As shown in Figure 1b, as the DODAC content was varied, an encapsulation profile for DOPE-containing systems similar to that obtained for the POPC-containing systems was



observed. Significant differences are that maximum encapsulation was greater (approximately 70%) for the DOPE-containing system and that optimum encapsulation was observed at about 6 mol% DODAC, compared with approximately 9% DODAC for the POPC-containing particles. If PEG-CerC₁₄ was substituted for PEG-CerC₂₀ very similar plasmid encapsulation behavior was observed.

In subsequent experiments DOPE/DODAC/PEG-Cer formulations were employed containing 6 mol% DODAC. For this fixed DODAC content, some batch-to-batch variability of encapsulation efficiency (typically over the range 50–70%) was observed when different batches of plasmid were employed. This variability resulted from small changes (up to ± 1 mol%) in the DODAC concentration required for maximum encapsulation efficiency for different plasmid batches. Other factors which may influence encapsulation efficiency include the amount of the plasmid present and the size of the plasmid. The plasmid (pCMVCAT) concentration was varied over the range 25 to 400 $\mu\text{g}/\text{ml}$ employing the DOPE/DODAC/PEG-CerC₂₀ (84:6:10; mol:mol:mol) lipid mixture at a fixed total lipid concentration of 10 mg/ml. Encapsulation efficiencies of more than 50% were achieved over this range (data not shown). In addition, at a plasmid concentration of 400 $\mu\text{g}/\text{ml}$, similar levels of entrapment were observed for plasmids of 4.49 and 10 kbp in length (data not shown).

It is important to show that the detergent dialysis process does not inhibit the transfection potential of the encapsulated plasmid. In order to test this, the plasmid was extracted from SPLP as described in Materials and methods. Characterization of the extracted DNA by agarose gel electrophoresis indicated no DNA degradation or plasmid relaxation relative to the starting material. Furthermore, the luciferase activity measured in cells following transfection (mediated by calcium phosphate) with plasmid extracted from SPLP was equivalent to the activity observed for plasmid which had not undergone encapsulation, with activities of 0.44 ± 0.15 ng and 0.35 ± 0.2 ng, respectively for 0.5 μg plasmid per well.

Plasmid DNA in stabilized plasmid-lipid particles is protected from DNase I and serum nucleases

It is important to demonstrate that the 'encapsulated' plasmid in the particles obtained by the detergent dialysis process is, in fact, fully protected from the external environment. As a first measure of protection, the ability of DNase I to digest plasmid DNA in DOPE-containing particles was examined. SPLP were prepared for the DOPE/DODAC/PEG-CerC₂₀ (84:6:10; mol:mol:mol) lipid mixture and pCMVLuc (200 $\mu\text{g}/\text{ml}$). Protection of plasmid in the SPLP was compared to protection of plasmid in complexes with DODAC-DOPE (1:1; mol:mol) LUVs and to free plasmid. Samples containing 1 μg plasmid were exposed to 0, 100 and 1000 units of DNase I for 30 min at 37°C. After incubation the plasmid was isolated as described in Materials and methods and characterized by agarose gel electrophoresis. As shown in Figure 2, free plasmid is completely digested by incubation with both 100 and 1000 units of DNase I. The plasmid complexed with cationic LUVs is somewhat protected compared with free DNA when exposed to 100 units of DNase I, but is almost entirely digested by incubation with 1000 units. In contrast, plasmid DNA in the SPLP is digested only when detergent is added to disrupt the SPLP before incubation with DNase I.

A rigorous test of SPLP stability and protection of encapsulated plasmid involves incubation in serum. Serum contains a variety of nucleases, and serum proteins can rapidly associate with lipid systems,¹⁶ resulting in enhanced leakage and rapid clearance of liposomal systems. The ability of serum nucleases to degrade plasmid is illustrated in Figure 3a. Intact pCMVCAT elutes in the void volume of the Sepharose CL-4B column, whereas after incubation with mouse serum (90%) at 37°C for 30 min the plasmid is degraded into fragments which elute in the included volume. The behavior of the DOPE/DODAC/PEG-CerC₂₀ (84:6:10; mol:mol:mol) SPLP system where non-encapsulated plasmid has not been removed is shown in Figure 3b. In this particular preparation, 53% of the plasmid DNA elutes with the lipid in the void volume and 47% of the DNA, which

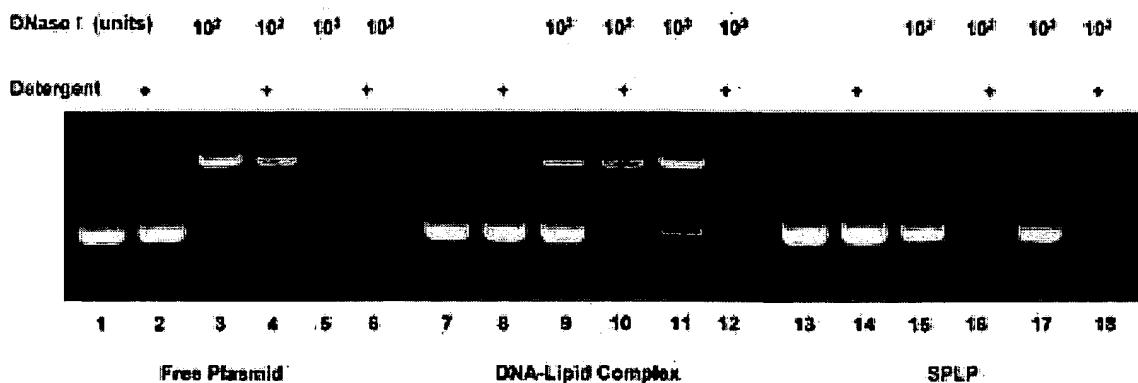


Figure 2 Stability of free plasmid, plasmid encapsulated in SPLP and plasmid in plasmid DNA-cationic lipid complexes in the presence of DNase I. Each of the sample types was subjected to six different protocols, giving rise to six lanes for each sample. These protocols consisted of no exposure to DNase I or detergent (lanes 1, 7, 13), exposure to detergent alone (lanes 2, 8, 14), exposure to 100 and 1000 units of DNase I alone (lanes 3, 9, 15 with 100 units and lanes 5, 11, 17 with 1000 units) and exposure to both detergent and DNase I (lanes 4, 10, 16 with 100 units and lanes 6, 12, 18 with 1000 units). These experiments utilized 1 μg of plasmid DNA (pCMVLuc), 1% Triton X-100 and 100 or 1000 units of DNase I. These components were combined in a total volume of 100 μl of 5 mM HBS and 10 mM MgCl₂, and incubated for 30 min at 37°C before preparation for gel electrophoresis as outlined in Materials and methods. The plasmid DNA-cationic lipid complexes were prepared as indicated in Materials and methods and consisted of DODAC:DOPE (50:50; mol:mol) LUVs (100 nm diameter) complexed to plasmid at a 3:1 charge ratio (positive-to-negative).

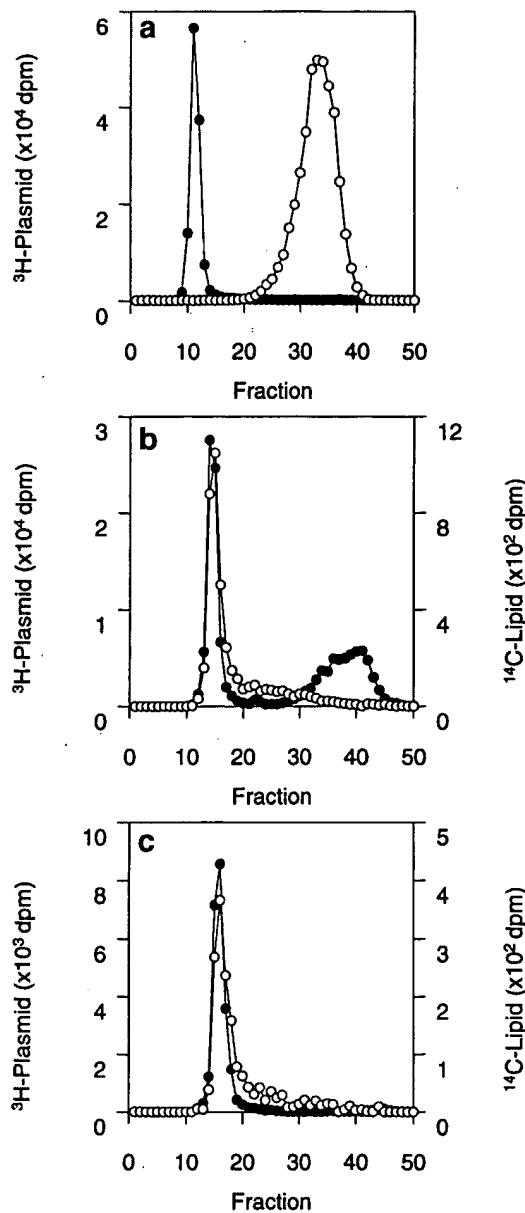


Figure 3 Plasmid in SPLP is protected from serum nuclease cleavage. The stability of plasmid (*pCMVcat*) in the free form or encapsulated in SPLP was determined in the presence of serum. The SPLP (DOPE/DODAC/PEG-CerC₂₀; 84:6:10; mol:mol:mol) were prepared as indicated in the legend to Figure 1 and contained ¹⁴C-labeled CHE as a lipid marker. Samples with 5 µg of ³H-labeled plasmid DNA were incubated in the presence of HBS or 90% mouse serum for 30 min at 37°C and eluted on a Sepharose CL-4B column equilibrated in HBS. (a) Elution profile of nucleic acid resulting from incubation of free plasmid in HBS (●) or 90% mouse serum (○). (b) Elution profile of nucleic acid (●) and lipid (○) following incubation of SPLP in 90% mouse serum. (c) Elution profile of nucleic acid (●) and lipid (○) following incubation of SPLP with mouse serum where unencapsulated plasmid was removed by anion exchange chromatography before the serum treatment.

represents degraded plasmid, elutes in the included volume. This indicates that 53% of the plasmid is encapsulated and protected from the external environment, in good agreement with a 55% trapping efficiency of this sample as determined by DEAE ion exchange chromatography.

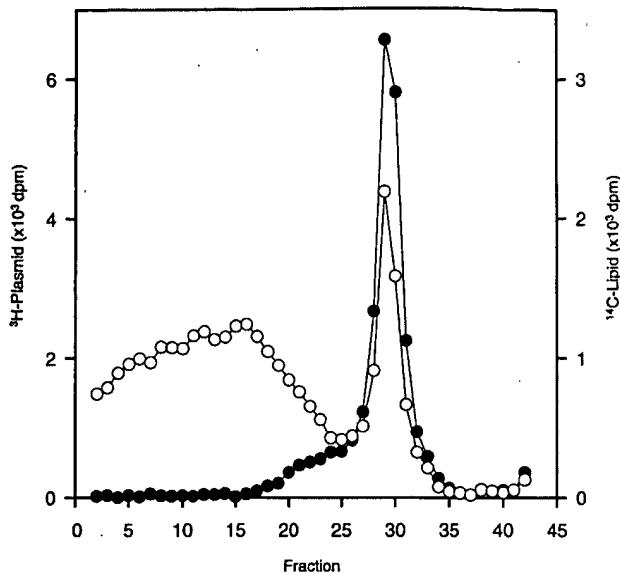


Figure 4 Separation of SPLP from empty vesicles by discontinuous sucrose density gradient centrifugation. The solid circles indicate the behavior of the ³H-labeled plasmid (*pCMVcat*), whereas the open circles indicate the distribution of lipid as reported by the ¹⁴C-labeled CHE lipid marker. SPLP (DOPE/DODAC/PEG-CerC₂₀; 84:6:10; mol:mol:mol) were prepared as indicated in the legend to Figure 1, and an aliquot (1.5 ml containing approximately 50 µg of ³H-plasmid DNA) was applied to a discontinuous sucrose density gradient (3 ml 10% sucrose, 3 ml 2.5% sucrose, 3 ml 1% sucrose; all in HBS). The gradient was then centrifuged at 160 000 g for 2 h.

A final test of the stability of the SPLP formulation is given in Figure 3c, which shows the elution profile of the DOPE/DODAC/PEG-CerC₂₀ (84:6:10; mol:mol:mol) SPLP system following removal of the external plasmid by DEAE chromatography and incubation in 90% mouse serum (30 min at 37°C). In this case more than 95% of plasmid applied to the column eluted in the void volume, demonstrating the stability and the plasmid protection properties of the SPLP formulation. It should also be noted that SPLP containing PEG-CerC₁₄, in place of PEG-CerC₂₀, exhibited similar plasmid protection properties.

Stabilized plasmid-lipid particles can be isolated by density centrifugation

The detergent dialysis process clearly results in plasmid-containing particles where the plasmid is protected from the external environment. However, it is likely that empty vesicles are also produced, as detergent dialysis of lipids (in the absence of plasmid) is well known to result in the formation of small lipid vesicles.¹⁷ These empty vesicles may be expected to be less dense than SPLP. The density gradient profile of a DOPE/DODAC/PEG-CerC₂₀ (84:6:10; mol:mol:mol) SPLP preparation (plasmid-to-lipid ratio of 200 µg DNA to 10 mg lipid) was therefore examined employing sucrose density step gradient centrifugation. As shown in Figure 4, after centrifugation at 160 000 g for 2 h, the encapsulated DNA is present as a band which was localized at the 2.5% sucrose-10% sucrose interface in the step gradient. It is interesting to note that less than 10% of the total lipid (as assayed by the ³H-CHE lipid marker) is associated with the plasmid DNA, which corresponds to 55% of the total DNA. The plasmid-to-lipid ratio in these

purified SPLP was determined (as indicated in Materials and methods) to be 62.5 µg plasmid per µmol lipid. It was found that SPLP generated by detergent dialysis and purified by density gradient centrifugation may be concentrated by dialysis against carboxymethyl cellulose to achieve plasmid concentrations of 1 mg/ml or higher.

Stabilized plasmid-lipid particles exhibit a narrow size distribution

The sizes of the empty lipid vesicles in the upper band and the isolated SPLP in the lower band of the sucrose density gradient were examined by quasi-elastic light scattering (QELS) and freeze-fracture electron microscopy techniques. As shown in Figure 5, the QELS analysis indicated that the mean diameter of the empty vesicles was approximately 44 nm ($\chi^2 = 0.48$), whereas the isolated SPLP were larger, with a mean diameter of 75 nm ($\chi^2 = 0.14$). Freeze-fracture electron microscopy studies gave similar results (Figure 6). A size analysis of the particles in these micrographs indicated a size of 36 ± 15 nm for the empty vesicles and 64 ± 9 nm for the isolated SPLP.

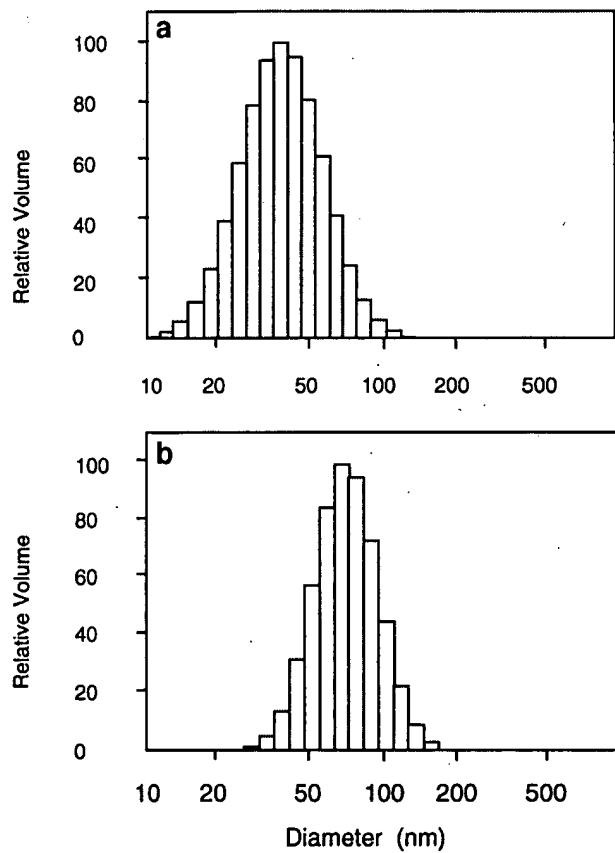


Figure 5 Size distribution of SPLP and empty vesicles as determined by QELS. SPLP were prepared containing pCMVLuc as indicated in the legend to Figure 1, and separated from empty vesicles by discontinuous sucrose density gradient centrifugation. (a) Size distribution for empty vesicles (upper band). (b) Size distribution for SPLP (lower band). The sizes were determined by quasi-elastic light scattering using a Nicomp (Santa Barbara, CA, USA) model 370 sub-micron particle sizer operating in the solid particle mode.

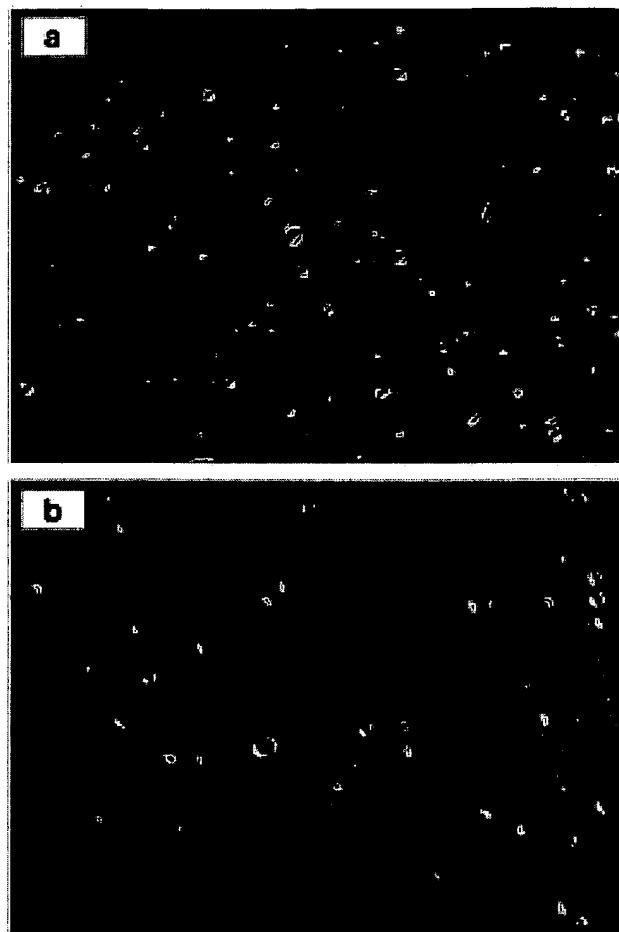


Figure 6 Freeze-fracture electron microscopy of purified SPLP and empty vesicles. SPLP containing pCMVLuc were prepared as indicated in the legend to Figure 1 and separated into (a) empty vesicles and (b) SPLP employing discontinuous sucrose density gradient centrifugation. The bar indicates 200 nm. For details of sample preparation and electron microscopy, see Materials and methods.

In vitro transfection properties of stabilized plasmid-lipid particles

SPLP consisting of DOPE/DODAC/PEG-CerC₂₀ (84:6:10) containing pCMVLuc coding for the luciferase reporter gene were prepared for transfection studies. As shown in Figure 7, after incubation of these SPLP with COS-7 cells for 24 h, little if any transfection activity was observed. It is probable that the presence of the PEG coating on the SPLP inhibits the association and fusion of the SPLP with cells in the same manner that PEG coatings inhibit fusion between lipid vesicles,¹³ and thus inhibit intracellular delivery of the encapsulated plasmid. In this regard, previous studies¹³ on LUVs with PEG coatings attached to phosphatidylethanolamine (PE) anchors have demonstrated that, for PE anchors containing short acyl chains, the PEG-PE can rapidly exchange out of the LUV, rendering the LUVs increasingly able to interact and fuse with each other. The transfection properties of SPLP containing PEG-CerC₂₀ were therefore compared to SPLP containing PEG-CerC₁₄, which has a shorter acyl chain. As shown in Figure 7, after incubation with COS-7 cells for 24 h, the SPLP containing PEG-CerC₁₄ exhibits substantially higher levels of transfection compared with the

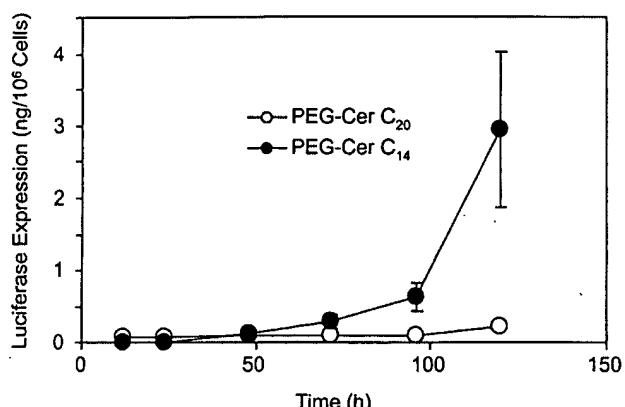


Figure 7 Effect of PEG-Cer coating of SPLP on transfection activity in vitro. Plasmid (*pCMV*Luc) was encapsulated in SPLP (DOPE/DODAC/PEG-Cer; 84:6:10, mol/mol/mol) containing PEG-CerC₂₀ (○) or PEG-CerC₁₄ (●). Non-encapsulated plasmid was removed by anion exchange chromatography, as indicated in Materials and methods. The SPLP preparation (1 µg plasmid) was then added to COS-7 cells at a density of 2×10^4 per 24-well plate. The cells were incubated with the SPLP for the times indicated, and luciferase activity was measured as indicated in Materials and methods.

system containing PEG-CerC₂₀. This is consistent with the ability of the PEG-CerC₁₄ coating to diffuse away from the SPLP surface. The SPLP containing either PEG-CerC₁₄ or PEG-CerC₂₀ exerted no apparent toxic effects on the cells as evaluated by monitoring protein content in the cell extract.

In order to determine whether the improved transfection properties of SPLP containing PEG-CerC₁₄ as compared with SPLP containing PEG-CerC₂₀ could be related to a faster dissociation rate from the SPLP surface, the dissociation rates at 37°C of radiolabeled PEG-CerC₁₄ and PEG-CerC₂₀ from 100 nm diameter large unilamellar vesicles (LUV) composed of egg phosphatidylcholine (EPC) were measured as indicated in Materials and methods. It should be noted that it is difficult to measure the dissociation rate of PEG-Cer from the surface of SPLP containing DOPE as the stability of these SPLP is dependent on the presence of the PEG-Cer coating. It was found that PEG-CerC₂₀ dissociated very slowly, with more than 90% remaining with the SPLP after 48 h incubation, corresponding to a half-time for dissociation of $t_{1/2} > 13$ days. In contrast, PEG-CerC₁₄ rapidly dissociated from the outer monolayer of the LUV with $t_{1/2} = 1.1 \pm 0.3$ h.

Discussion

This study presents a new method of encapsulating plasmid DNA in small, stable particulate systems that may find utility as gene delivery vehicles. Of particular interest are the relationship between properties of SPLP and other lipid-based systems containing plasmids, the structure of SPLP and the potential utility of SPLP with exchangeable PEG coatings. We discuss these areas in turn.

The SPLP protocol for plasmid entrapment allows trapping efficiencies of up to 70% and results in stable particles containing low levels of cationic lipids and high levels of fusogenic lipids, such as DOPE. These particles are small (<100 nm diameter), are resistant to external nucle-

ases, exhibit high DNA-to-lipid ratios (62.5 µg/µmol) and can be concentrated to achieve high plasmid DNA concentrations (1 mg/ml). Furthermore, the detergent dialysis procedure is a gentle procedure that results in little, if any, plasmid degradation.

These features of SPLP contrast favorably with previous plasmid encapsulation procedures. Plasmid DNA has been encapsulated by a variety of methods, including reverse phase evaporation,^{18–20} ether injection,^{21,22} detergent dialysis in the absence of PEG stabilization,^{20,21} lipid hydration and dehydration-rehydration techniques^{25–27} and sonication,^{28–30} among others. The characteristics of these protocols are summarized in Table 1. None of these procedures yields small, serum-stable particles at high plasmid concentrations and plasmid-to-lipid ratios in combination with high plasmid-encapsulation efficiencies. Trapping efficiencies comparable with the SPLP procedure can be achieved employing methods relying on sonication. However, sonication is a harsh technique which can shear nucleic acids.³¹ Size ranges of 100 nm diameter or less can be achieved by reverse phase techniques; however, this requires an extrusion step through filters with 100 nm or smaller pore size which can often lead to significant loss of plasmid. Finally, it may be noted that the plasmid DNA-to-lipid ratios that can be achieved for SPLP are significantly higher than those achievable by any other encapsulation procedure.

With regard to the structure of SPLP, any model must take into account two important observations. First, SPLP form only at a critical cationic lipid content of approximately 6 mol%. At higher cationic lipid contents, aggregation is observed, whereas lower cationic lipid contents lead to little or no plasmid encapsulation. Second, purified SPLP exhibit a plasmid DNA-to-lipid ratio of 62.5 µg/µmol. For a 4.49 kbp (*pCMVCAT*) plasmid, this corresponds to a plasmid-to-particle ratio of 0.97 for an SPLP diameter of 70 nm (the average of the freeze-fracture electron microscopy and QELS results), assuming a lipid molecular area³² of 0.67 nm² and an average nucleotide molecular weight of 330. It may therefore be concluded that SPLP contain one plasmid per particle.

The model that guided the construction of SPLP relied on the hypothesis that the plasmid combines with the cationic lipid to form a hydrophobic ‘inverted micellar’ structure that is stabilized in aqueous media by the detergent. In this model the addition of DOPE and PEG-Cer and subsequent dialysis results in deposition of a monolayer of DOPE and PEG-Cer around the hydrophobic intermediate, resulting in a stabilized plasmid-lipid particle. It is instructive to perform some simple calculations to see whether this model is consistent with experimental observations. In particular, if each negative charge on the plasmid has a cationic lipid associated with it, the total volume of each hydrophobic plasmid-cationic lipid particle can be calculated to be approximately 1.35×10^4 nm³ for a 4.49 kbp plasmid. This calculation assumes that plasmid DNA has a density of 1.7 g/ml, the molecular weight of each base is 330, and that, as an upper limit, the volume per molecule of the cationic lipid is 1.5 nm³, which is the volume of a liquid crystalline bilayer-forming lipid such as dioleoylphosphatidylcholine (lipid length 2.2 nm and area per molecule 0.67 nm²).³² Thus, if each SPLP contained one *pCMVCAT* plasmid completely neutralized by associated cationic lipid and arranged in a spherical conformation, the predicted diameter would

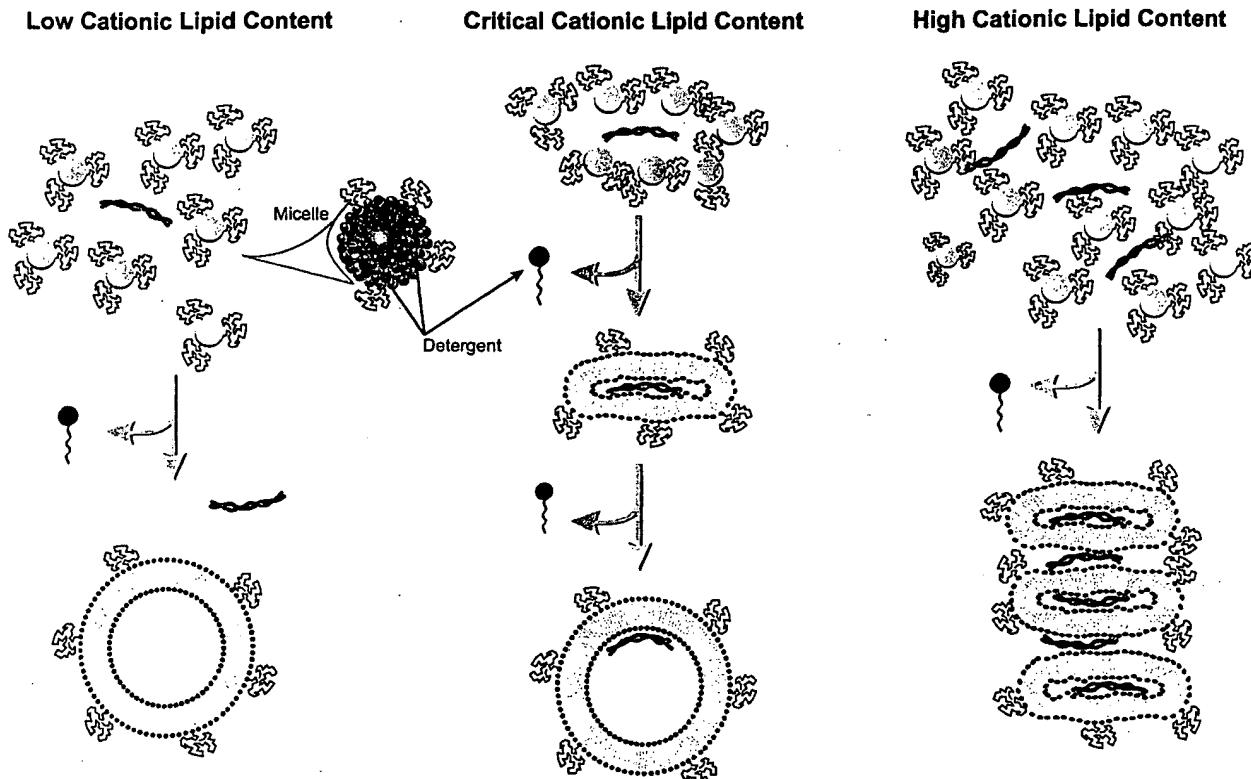


Figure 8 Model of the formation and possible structure of SPLP. The first stage of dialysis is proposed to result in formation of macromolecular lipid intermediates, which may be in the form of lamellar sheets, cylindrical micelles or leaky vesicles.^{33,34} If the cationic lipid content is too low (left panel), plasmid does not associate with these intermediates as dialysis proceeds, leading to formation of empty vesicles and free plasmid. At higher cationic lipid contents plasmid associates with the lipid intermediates, drawn here as a bilayer sheet wrapped around the plasmid. If the cationic lipid content is at a critical level the presence of the plasmid reduces the net positive surface charge of the lipid intermediate to the extent that further association of plasmid is inhibited. As dialysis proceeds further, additional lipid would be expected to condense on this structure, leading to formation of a vesicle containing encapsulated plasmid, as indicated. In addition, empty vesicles and free plasmid would be expected. At high cationic lipid contents (right panel), the surface charge on the lipid intermediate structures is so high that two or more plasmids can associate with a given membrane sheet, leading to the formation of large aggregates.

be approximately 30 nm. The freeze-fracture electron microscopy results presented here indicate that SPLP containing the pCMVCAT plasmid exhibit a diameter of approximately 70 nm, and are therefore too large to be composed solely of a plasmid-lipid particle with no interior aqueous volume.

An alternative working model for SPLP formation and structure is shown in Figure 8. It is unlikely that plasmid associates directly with the micelles, as the presence of high levels of detergent may be expected to dilute the positive surface charge due to the cationic lipid to the extent that electrostatic association is reduced. A probable first step of the dialysis process is the formation of macromolecular lipid intermediates, which may include cylindrical micelles, lamellar sheets or leaky vesicles that form as detergent is removed. These structures have been observed as intermediates in the micelle to vesicle transition undergone by dispersions of egg phosphatidylcholine as detergent (OGP) is removed by dialysis.^{33,34} These structures are represented in Figure 8 as lamellar sheets by way of example. As shown in the left panel of Figure 8, low concentrations of cationic lipid would result in little association of plasmid with these intermediate structures, which is consistent with little or no plasmid

entrapment following detergent dialysis. At high concentrations of cationic lipid, intermediate structures may be expected to associate with the plasmid and, if the cationic lipid content is too high, plasmid-lipid-plasmid association could dominate as dialysis proceeds, leading to formation of aggregates (Figure 8, right panel).

If the cationic lipid content is at a critical level (Figure 8, central panel), the positive surface charge on the plasmid-associated intermediates will be reduced below that needed to associate with other plasmids, due to charge neutralization. This would mitigate against further aggregation. Further dialysis will result in fusion between intermediates eventually to produce empty vesicles or in fusion between intermediates and the plasmid-lipid particle. Fusion with the particle will result in the deposition of excess bilayer lipid, leading to the formation of an associated vesicle in the final SPLP. In the structure presented, the plasmid is associated with the inner monolayer of the vesicle that is produced as more lipid is deposited in the particle. It should be noted that the forces driving a partial removal of the plasmid lipid coat are not clear, and it is possible that the plasmid resides in a hydrophobic domain inside the particle.

The final area of discussion concerns the potential util-

Table 1 Procedures for encapsulating plasmid in lipid-based systems

Procedure	Lipid composition	Length of DNA	Trapping efficiency ^a	DNA-to-lipid-ratio ^b	Diameter
Reverse phase evaporation ¹⁸	PS or PS:Chol (50:50)	SV40 DNA	30–50%	<4.2 µg/µmol	400 nm
Reverse phase evaporation ¹⁹	PC:PS:Chol (40:10:50)	11.9 kbp plasmid	13–16%	0.23 µg/µmol	100 nm to 1 µm
Reverse phase evaporation ²⁰	PC:PS:Chol (50:10:40)	8.3 kbp, 14.2 kbp plasmid	10%	0.97 µg/µmol	ND
Reverse phase evaporation ⁴¹	EPC:PS:Chol (40:10:50)	3.9 kbp plasmid	12%	0.38 µg/µmol	400 nm
Ether injection ²¹	EPC:EGP (9:1)	3.9 kbp plasmid	2–6%	<1 µg/µmol	0.1–1.5 µm;
Ether injection ²²	PC:PS:Chol (40:10:50)	3.9 kbp plasmid	15%	15 µg/µmol	ND
PC:PG:Chol (40:10:50)					
EPC:Chol/stearylamine (43:5:5:43:5:13)	sonicated genomic DNA (250 000 mw)		11%	0.26 µg/µmol	50 nm
DOPC:Chol:oleic acid or, DOPE:Chol:oleic acid (40:40:20)	4.6 kbp plasmid		14–17%	2.25 µg/µmol	180 nm (DOPC)
EPC:Chol (65:35) or EPC			ND		290 nm (DOPE)
Dehydration-rehydration extrusion (400 or 200 nm filters) ²³	3.9 kbp, 13 kbp plasmid ND				0.5–7.5 µm
EPC	2.96 kbp, 7.25 kbp plasmid 1.0 kbp linear DNA		35–40%	2.65–3.0 µg/µmol	1–2 µm
Sonication (in the presence of lysozyme) ²⁴			50%	0.08 µg/µmol	100–200 nm
Sonication ²⁵	EP:Chol:Lysine-DPPE (55:30:15)	6.3 kb ssDNA 1.0 kb dsRNA	60–95% ssDNA; 80–90% dsRNA	13 µg/µmol ssDNA; 14 µg/µmol dsRNA	100–150 nm
Spermidine-condensed DNA, sonication, extrusion ³⁰	EP:Chol:PS (40:30:10) EP:Chol:EEPA (40:50:10)	4.4 kbp, 7.2 kbp plasmid	46–52%	2.53–2.87 µg/µmol	400–500 nm
Ca ²⁺ -EDTA entrapment of DNA- protein complexes ⁴²				22 µg/µmol	ND
Freeze-thaw, extrusion ⁴³	POPC:DDBB (99:1)	42.1 kbp bacteriophage	52–59%		
SPLP (this work)	DOPE:PEG-Cer:DODAC (84:10:6)	3.4 kbp linear plasmid 4.4–10 kbp plasmid	17–50% 60–70%	62.5 µg/µmol	80–120 nm 75 nm (QELS); 65 nm (freeze-fracture)

^aSome values calculated based on presented data.
^bND, not determined.

ity of SPLP with exchangeable PEG coatings. As previously indicated, the SPLP system has been designed for systemic (intravenous) gene therapy applications. This places two potentially conflicting demands on the delivery system. First, the carrier must circulate long enough to achieve accumulation at disease sites, such as tumors, by taking advantage of the increased vascular permeability in these regions. Second, the carrier must be able to bind to target cells and to destabilize the plasma or endosomal membrane after arrival at the disease site in order to facilitate intracellular delivery of the enclosed plasmid. The first requirement implies a very stable carrier that does not interact with cells, whereas the second requirement necessitates a particle that can bind to cells and exhibit a membrane-destabilizing 'fusogenic' character.

PEG coatings that can dissociate from a carrier provide a potential solution to these demands. First, the presence of a PEG coating allows SPLP to be formed with a large proportion of DOPE in the outer monolayer. Previous work has shown that DOPE prefers the (non-bilayer) hexagonal H_2 phase at temperatures above 10°C,³⁵ and that PEG lipids can stabilize DOPE in the bilayer organization.³⁶ Thus in the absence of the PEG-Cer the SPLP would be expected to be highly unstable and fusogenic. The detergent dialysis procedure therefore allows an intrinsically fusogenic plasmid-containing particle to be formed, where the stability of the particle is dependent on the presence of the PEG coating. As demonstrated here, these particles are stable in the presence of DNase I, as well as serum nucleases, consistent with an ability to protect encapsulated DNA in the circulation. In addition, the small size and presence of the PEG coating would be expected to promote the extended circulation life-times required to achieve preferential accumulation at disease sites such as tumors following intravenous administration.

The stability of the SPLP would, however, be expected to mitigate against uptake and intracellular delivery of the plasmid. The use of PEG coatings that dissociate from the SPLP after arrival at a disease site provides a potential solution to this problem. This is supported by the *in vitro* results presented here, which show that a PEG-CerC₂₀ coating, which has a long residence time in lipid bilayers, exhibits poor transfection properties, whereas improved transfection is observed for the SPLP containing a PEG-CerC₁₄ coating, which can dissociate from lipid bilayers more rapidly.

In summary, this study presents a method for encapsulating plasmid DNA in particulate systems that have the properties of small size, high plasmid-to-lipid ratio and high content of fusogenic lipid, and that can be concentrated to achieve high plasmid concentrations. These SPLP are stabilized by the presence of a PEG coating that can be designed to dissociate, thus increasing the transfection potency of the SPLP. It is expected that these systems will find utility as delivery systems for systemic gene therapy.

Materials and methods

Materials

Dioleoylphosphatidylethanolamine (DOPE) was obtained from Northern Lipids (Vancouver, BC, Canada). The

lipids 1-O-(2'-(ω -methoxypolyethyleneglycol) succinyl)-2-N-myristoylsphingosine (PEG-CerC₁₄) and 1-O-(2'-(ω -methoxypolyethyleneglycol) succinyl)-2-N-arachidoylsphingosine (PEG-CerC₂₀) were synthesized as described elsewhere,³⁷ and dioleyldimethylammonium chloride (DODAC) was kindly provided by Dr S Ansell (Inex Pharmaceuticals). Octylglucopyranoside (OGP), HEPES and NaCl were obtained from Sigma (St Louis, MO, USA). The plasmid pCMVCAT (4490 bp, coding for the chloramphenicol acyl transferase gene) was originally obtained from Dr K Brigham (Vanderbilt University, Nashville, TN, USA). The plasmid pCMVLuc (5650 bp, coding for the luciferase reporter gene) was provided by Dr P Tam (Inex Pharmaceuticals). All reporter genes were under the control of the human CMV immediate-early promoter-enhancer element. ³H-cholesteryl hexadecyl ether (CHE) and ¹⁴C-CHE were obtained from Mandel Scientific (Guelph, ON, Canada). Mouse serum was obtained from CedarLane (Mississauga, ON, Canada). Dialysis tubing (Spectrapor 12 000 to 14 000 mwco) was obtained from Fisher Scientific (Ottawa, ON, Canada), DEAE-Sepharose CL-6B column from Sigma, *E. coli* DNase I from Life Technologies (Mississauga, ON, Canada) and the luciferase assay kit from Promega (Madison, WI, USA).

Preparation of plasmids

Plasmid DNA was transformed into *E. coli* strain DH5α by electroporation. Plasmid DNA was then isolated from *E. coli* by alkaline lysis³⁸ followed by anion exchange chromatography (according to the manufacturer, Qiagen, Santa Clarita, CA, USA) or CsCl gradient centrifugation.³⁹ DNA was precipitated and dissolved in pyrogen-free water for formulation with lipids.

Radiolabeled ³H-plasmid DNA was isolated from an *E. coli* JM101 strain bearing pCMVβ, pCMVCAT or pCMVLuc. Briefly, cultures were grown in supplemented minimal media (M9 salts with 0.1% thiamine, 1% glucose, 100 µg/ml ampicillin) to mid log phase. Ten mCi of 81.9 mCi/mmol tritiated thymidine (Mandel Scientific) was added, then the cultures were allowed to grow for a further 12–16 h. Plasmid DNA was isolated by alkaline lysis and anion exchange chromatography, as described above.

Encapsulation of plasmid DNA

Plasmid DNA (50–400 µg) was incubated with DODAC in 500 µl of 0.2 M octylglucoside, 150 mM NaCl, 5 mM HEPES pH 7.4 for 30 min at room temperature. The plasmid-DODAC mixture was then added to DOPE and PEG-CerC₁₄ or PEG-CerC₂₀ dissolved in 500 µl of 0.2 M OGP; 150 mM NaCl, 5 mM HEPES pH 7.4. The total lipid concentration was either 5 or 10 mg/ml with DOPE:DODAC:PEG-Cer at molar ratios of 84:6:10, unless otherwise indicated. The plasmid-lipid mixture was dialyzed against 5 mM HEPES in 150 mM NaCl pH 7.4 (HBS) for 36 to 48 h with two buffer changes. Non-encapsulated plasmid was removed by anion exchange chromatography on a DEAE-Sepharose CL-6B column (1 × 4 cm). To determine the encapsulation efficiency, a 50-µl aliquot of each sample was loaded on to a DEAE-Sepharose CL-6B column (1 ml) equilibrated with HBS. The column was eluted with HBS and the fractions were assessed for ³H-plasmid and ¹⁴C-lipid by scintillation counting.

Isolation of encapsulated plasmid by sucrose density gradient centrifugation

The fractions from the DEAE column containing co-eluting lipid and plasmid were pooled and equal volumes were applied to the top of a discontinuous sucrose gradient in 12.5 ml ultracentrifuge tubes. The gradient was formed with 3 ml each of 10% sucrose, 2.5% sucrose and 1% sucrose in HBS layered consecutively from bottom to top. The gradients were centrifuged at 160 000 g for 2 h at 20°C and separated into aliquots (250 µl) removed from top to bottom. The fractions were assayed for ³H-plasmid and ¹⁴C-CHE by dual-label scintillation counting. The lipid encapsulated plasmid DNA banded tightly at the interface between 2.5% and 10% sucrose, while the unassociated lipid was present as a smear from the top of the gradient to the interface between 1% and 2.5% sucrose. It was found that the isolated SPLP could be concentrated by dialysis against 500 000 molecular weight carboxymethyl cellulose (Aquacide II; Calbiochem, San Diego, CA, USA) in a 12 000–14 000 molecular weight cut-off dialysis tube. When the desired volume was reached, the formulation was transferred into a new dialysis bag and dialyzed overnight against HBS to adjust the NaCl concentration to 150 mM.

Freeze-fracture electron microscopy

Freeze-fracture was performed on a Balzers Freeze-Etching system, BAF 400D (Balzers, Lichtenstein). Samples were cryofixed in the presence of 25% glycerol by plunging them into liquid freon 22. The fractured surface was shadowed unidirectionally with platinum/carbon (45°) and coated with carbon (90°) immediately after fracturing. Replicas were analyzed using a Jeol model JEM 1200 EX electron microscope (Jeol, Montreal, QC, Canada).

Serum stability assay

SPLP formulations were assayed for serum stability in the presence of 90% mouse serum *in vitro*. A 50 µl aliquot was added to 450 µl mouse serum and incubated at 37°C for 30 min. The sample was then loaded on to a Sepharose CL-4B column and eluted with HBS, pH 7.4. The fractions were analyzed for ³H-plasmid and the lipid label ¹⁴C-CHE.

Determination of DNase I stability

Three sets of samples were exposed to DNase I digestion, including naked plasmid DNA, plasmid complexed with DOPE:DODAC vesicles and SPLP. Plasmid–cationic lipid complexes were prepared by mixing 500 µl plasmid (pCMVLuc, 0.5 mg/ml) in 5% glucose with 500 µl DODAC:DOPE (1:1) 100 nm diameter LUVs (0.9 mM lipid) prepared by the extrusion method⁴⁰ in 5% glucose. This corresponds to a lipid-to-DNA charge ratio (positive-to-negative) of 3. The resulting solution was incubated at room temperature for 30 min before DNase I treatment. For the DNase digestion, samples (free plasmid, plasmid–lipid complex, encapsulated plasmid) containing 1 µg of DNA were incubated with 0, 100 or 1000 units of DNase I in a total volume of 100 µl of 5 mM HEPES, 150 mM NaCl, 10 mM MgCl₂ pH 7.4 in the presence or absence of 1.0% Triton X-100. After incubation at 37°C for 30 min, the DNA was isolated by adding 500 µl of DNAzol (Life Technologies) followed by 1.0 ml of ethanol. The samples were centrifuged for 30 min at 20 000 g in a tabletop microfuge. The supernatant was decanted and the DNA

pellet was washed twice with 80% ethanol and dried. The DNA was dissolved in 30 µl of TE buffer and analyzed by agarose (1.0%) gel electrophoresis in TAE buffer.

In vitro transfection

COS-7 cells and 293 cells were grown at 37°C, 5% CO₂ in complete media consisting of T75 flasks in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) and 10% fetal bovine serum (FBS; Intergen, Purchase, MA, USA). Transfections were performed in the presence of cell culture media when the cells were 60–70% confluent. The plasmid (pCMVLuc) formulations were diluted in complete medium to give 0.5 µg DNA/ml. The cells were incubated in the presence of the plasmid formulations for up to 120 h and assayed for luciferase activity. Calcium phosphate-mediated transfection with plasmid extracted from SPLP was performed as follows. Plasmid (0.1–1 µg) in 50 µl 0.25 M CaCl₂ was slowly added to 50 µl HBS, and the resulting precipitate was added to 293 cells. Following incubation for 2 days at 37°C, the luciferase activity was determined.

Luciferase assay

Luciferase assays were performed using the Promega Luciferase Assay System reagent kit (Promega E1501) according to the manufacturer's instructions. Cell lysates were assayed for luciferase activity using a Dynex Technologies ML3000 microplate luminometer (Dynex Technologies, Ghentilly, VT, USA). Luminescence readings were calibrated according to a standard curve obtained using a *Photinus pyralis* luciferase standard (Boehringer Mannheim, Laval, QC, Canada; 634 409).

PEG-Cer dissociation rates

The dissociation rates of ³H-PEG-CerC₁₄ and ³H-PEG-CerC₂₀ from EPC LUV using EPC multilamellar vesicles (MLV) as a 'sink'. The LUV were prepared containing 10 mol% PEG-Cer and a trace of ¹⁴C-CHE (³H/¹⁴C ratio approximately 5) by detergent dialysis as described above for SPLP. MLV were prepared by hydration of EPC in HBS (250 mg/ml) at 65°C. The MLV were washed five times in HBS by centrifugation (2 min at 12 000 g) to remove any small vesicles. LUV (1 mg lipid) were mixed with 125 mg MLV to give a final volume of 1.5 ml and incubated at 37°C. At different time intervals, 100 µl of the mixture were transferred into 0.5 ml ice-cold HBS and the MLV pelleted by centrifugation. The LUV in the supernatant were analyzed for ³H-PEG-Cer and ¹⁴C-CHE and the ³H/¹⁴C ratio plotted as a function of time.

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Stabilized Plasmid-Lipid Particles: Pharmacokinetics and Plasmid Delivery to Distal Tumors following Intravenous Injection

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A previous study has shown that plasmid DNA can be encapsulated in lipid particles (SPLP, "stabilized plasmid lipid particles") of approximately 70 nm diameter composed of 1,2-dioleoyl-3-phosphatidyl-ethanolamine (DOPE), the cationic lipid N,N-dioleoyl-N,N-dimethylammonium chloride (DODAC) and poly(ethylene glycol) conjugated to ceramide (PEG-Cer) using a detergent dialysis process (Wheeler *et al.* (1999) *Gene Therapy* 6, 271-281). In this work we evaluated the potential of these SPLPs as systemic gene therapy vectors, determining their pharmacokinetics and the biodistribution of the plasmid and lipid components. It is shown that the blood clearance and the biodistribution of the SPLPs can be modulated by varying the acyl chain length of the ceramide group used as lipid anchor for the PEG polymer. Circulation lifetimes observed for SPLPs with PEG-CerC₁₄ and PEG-CerC₂₀ were $t_{1/2} = \sim 1$ and ~ 10 h, respectively. The SPLPs are stable while circulating in the blood and the encapsulated DNA is fully protected from degradation by serum nucleases. The accelerated clearance of SPLPs with PEG-CerC₁₄ is accompanied by increased accumulation in liver and spleen as compared to PEG-CerC₂₀ SPLPs. Delivery of intact plasmid to liver and spleen was detected. Significant accumulation (approximately 10% of injected dose) of the long circulating SPLPs with PEG-CerC₂₀ in a distal tumor (Lewis lung tumor in the mouse flank) was observed following iv application and delivery of intact plasmid to tumor tissue at approximately 6% injected dose/g tissue is demonstrated.

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Abbreviations: CHE, cholesteryl hexadecyl ether; Chol, cholesterol; DODAC, N,N-dioleoyl-N,N-dimethylammonium chloride; DOPE, 1,2-sn-dioleoyl-3-phosphatidyl-ethanolamine; POPC, 1-palmitoyl, 2-oleoyl-sn-phosphatidylcholine; EPC, egg phosphatidylcholine; HBS, 20 mM HEPES in 150 mM NaCl pH 7.4; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LUV, large unilamellar vesicle(s); MWCO, molecular weight cutoff; OGP, octylglucopyranoside; PEG-Cer_{C₁₄}, 1-O-(Z-(w-methoxypolyethyleneglycol) succinoyl)-2-N-myristoylsphingosine; PEG-Cer_{C₂₀}, 1-O-(Z-(w-methoxypolyethyleneglycol) succinoyl)-2-N-arachidoylsphingosine; PS, phosphatidylserine; SPLP, stabilized plasmid lipid particles; TAE, electrophoresis buffer (40 mM Tris acetate pH 8.5; 2 mM EDTA); RES, reticuloendothelial system; TE buffer, 10 mM Tris-Cl pH 8.0 and 1 mM EDTA

INTRODUCTION

The limitations of the currently available gene delivery systems for systemic application are widely recognized. Viral systems are rapidly cleared from the circulation limiting potential target sites to "first-pass" organs such as lung, liver and spleen. In addition, these systems elicit immune responses compromising the effectiveness of subsequent injections (Worgall *et al.*, 1997). The most common non-viral delivery system, the plasmid DNA-cationic lipid complexes (lipoplexes) carry an overall positive charge which results in rapid clearance from the circulation by the reticuloendothelial system (RES) following systemic administration *in vivo* (Mori *et al.*, 1998) limiting potential transfection sites to these "first-pass" organs (Hofland *et al.*, 1997; Lew *et al.*, 1995; Templeton *et al.*, 1997; Thierry *et al.*, 1995; Huang and Li, 1997). Furthermore, lipoplexes tend to form large aggregates ($\geq 300\text{nm}$ in diameter) which become trapped in the capillary beds of the lung (Yang and Huang, 1998). Therefore, the highest expression levels are usually observed in the lung (Hofland *et al.*, 1997; Barron *et al.*, 1998; Hong *et al.*, 1997; Felgner *et al.*, 1995), while expression levels in other organs are typically more than one magnitude lower. In addition, the bioavailability of the plasmid is compromised since a large amount of the plasmid DNA in lipoplexes is degraded in the blood due to cleavage by serum nucleases (Wheeler *et al.*, 1999).

The characteristics of lipoplexes are potentially useful for gene delivery to lung tissue, for example,

but are clearly unsuitable for delivery to a distal tumor site. Pharmacokinetic studies of liposomes have shown that small $\sim 100\text{ nm}$ diameter liposomes, which exhibit an extended circulation half-life accumulate preferentially at sites of disease including inflammation, infection and tumors. Such liposomes evade rapid removal from the circulation *in vivo* due to their small size and low surface charge (Allen and Chonn, 1987; Allen *et al.*, 1989; Gabizon and Papahadjopoulos, 1988; 1992). Inclusion of a PEG coating can also enhance the circulation lifetimes considerably. As much as 10% of the injected dose/g of tumor tissue of liposomes with PEG coating has been detected in tumors following systemic application (Allen and Chonn, 1987; Gabizon and Papahadjopoulos, 1992). This suggests that a small ($\sim 100\text{ nm}$ diameter) lipid-DNA particle with a PEG coating, in which the DNA is protected or hidden from the membrane surface and which presents an overall low surface charge should have the characteristics necessary for delivery to a tumor site *in vivo*. In this regard it was recently shown that plasmid DNA can be encapsulated in small stabilized lipid particles (SPLP) by a detergent dialysis method (Wheeler *et al.*, 1999).

Here, we determined the pharmacokinetics of these SPLPs following iv application. Clearance from the blood and biodistribution of SPLPs is shown in two normal mouse strains (CD-1 and BDF-1) and in BDF-1 tumor-bearing mice. The pharmacokinetics can be modulated by varying the lipid composition. Protection of the plasmid in the SPLP from degradation by serum nucleases in

the blood is demonstrated. Accumulation in first-pass organs particularly in the lung is significantly reduced with SPLPs that exhibit circulation longevity. Most importantly it is shown that iv administration of SPLPs results in delivery of high levels of intact plasmid to a distal tumor site.

MATERIALS AND METHODS

Materials

Dioleoylphosphatidylethanolamine (DOPE) was purchased from Avanti Polar Lipids Inc. (AL, USA). The lipids 1-O-(2'-(w-methoxypolyethylenglycol) succinoyl)-2-N-myristoylsphingosine (PEG-CerC₁₄) and 1-O-(2'-(w-methoxypolyethylenglycol) succinoyl)-2-N-arachidoylsphingosine (PEG-CerC₂₀) were synthesized as described elsewhere (Webb *et al.*, 1998), and dioleyldimethylammonium chloride (DODAC) was kindly provided by Dr. S. Ansell (Inex Pharmaceuticals Corp.). ³H and ¹⁴C labeled cholesteryl hexadecyl ether (³H-CHE and ¹⁴C-CHE) were purchased from Mandel Scientific (Guelph, Ont., Canada). Octylglucopyranoside (OGP), DEAE-Sepharose CL-6B, HEPES and NaCl were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The plasmid pCMVCAT (4490 bp, coding for the chloramphenicol acyl transferase gene) under the control of the human CMV immediate early promoter-enhancer element was originally obtained from Dr. K. Brigham (Vanderbilt University, Nashville, TN, USA). The plasmid was prepared as previously described (Birnboim and Doly, 1979; Sambrook *et al.*, 1989a) and the supercoiled plasmid isolated on a cesium chloride gradient and dialyzed against ultrapure distilled H₂O. The DNA was precipitated and dissolved in pyrogen-free water (1 mg/ml) for formulation with lipids. Mouse serum was obtained from CedarLane (Mississauga, Ont., Canada). Dialysis tubing (SpectraPor 12,000–14,000 mwco) was obtained from Fisher Scientific (Ottawa, Ont., Canada), PicoGreen™ from Molecular Probes (Eugene, OR, USA). All other chemicals were reagent grade.

Preparation of Lipid-DNA Complexes

Vesicles of 100 nm diameter composed of DOPE/DODAC (50 : 50 mol%) were prepared by an extrusion method described elsewhere (Hope *et al.*, 1985). Briefly, lipids dissolved in CHCl₃ were combined (5mg total lipid) and dried to a lipid film under a stream of nitrogen gas. Any remaining CHCl₃ was removed by lyophilization. The lipid was hydrated in 1 ml of HEPES Buffered Saline solution (HBS; 20 mM HEPES, 150 mM NaCl, pH 7.4), subjected to 5 freeze-thaw cycles and extruded through two 100 nm pore diameter polycarbonate filters. Lipid-DNA complexes were then prepared by mixing the appropriate quantity of plasmid (pCMVCAT) with the lipid vesicles to give a charge ratio (±) of 1:1.

Preparation and Isolation of SPLPs by Detergent Dialysis

The SPLPs were prepared by a detergent dialysis method as outlined previously (Wheeler *et al.*, 1999). Briefly, DODAC and the "other lipid" species (DOPE, Chol and PEG-ceramide, C14 : 0 or C20 : 0) were aliquotted into two separate test tubes from benzene : methanol (95 : 5) stock solutions to give the desired lipid ratio (5 or 10 mg/ml final lipid concentration) and the solvent removed by freeze-drying under high vacuum. Plasmid DNA (200 µg) was added to DODAC dissolved in 500 µl 0.2 M octylglucopyranoside (OGP) in HBS (20 mM HEPES in 150 mM NaCl pH 7.4). The "other lipids" were dissolved in 500 µl 0.2 M OGP in HBS and mixed with the DODAC-DNA suspension. The volume was adjusted to 1 ml with HBS and the mixture was dialyzed against 2 l HBS for 48 h with two buffer changes. Non-encapsulated plasmid was removed by anion exchange chromatography (DEAE-Sepharose CL-6B column, 1 x 4cm). The sample was eluted with HBS, placed in a dialysis bag and embedded in aquaice to concentrate to the desired volume. Following concentration the sample was dialyzed overnight against HBS to adjust the salt concentration. ³H-CHE, ¹⁴C-CHE

and ^3H -DNA were added as lipid and plasmid markers where appropriate.

Animal Studies

The SPLP preparations (200 μl) were injected into the lateral tail vein of CD-1 or BDF-1 mice and the animals sacrificed by CO_2 suffocation at the desired time points post-injection. Blood was collected into microtainer tubes following cardiac puncture, organs were harvested and tissues were homogenized by procedures described elsewhere (Parr *et al.*, 1997).

Lewis lung cells were grown in tissue culture using methods described elsewhere (Parr *et al.*, 1997). Approximately 300,000 Lewis lung carcinoma cells (ATCC CRL- 1642) were injected subcutaneously into the left hind flank of BDF-1 mice and tumors were allowed to grow for 14 days. The appropriate formulations (200 μl) were then injected into the lateral tail vein and the animals sacrificed at the desired time points post-injection. Blood collection and tissue harvesting were performed as described above.

Analysis of Lipid and Plasmid Biodistribution

Tissue homogenates were analyzed for ^3H -plasmid and ^{14}C -Lipid. Aliquots of tissue homogenates (200 μl of liver; 300 μl of heart, lung and kidney; 400 μl of spleen and 200 μl of tumor as appropriate) were placed in scintillation vials and 500 μl Solvable (Packard, Meriden, CT) added to each vial. Following overnight incubation at 60°C 500 μl distilled H_2O was added to the samples with vortex mixing and the color was bleached by addition of up to 200 μl hydrogen peroxide. Scintillation cocktail (5 ml Picofluor; Packard, Meriden, CT) was added to the sample, incubated at room temperature in the dark overnight and counted the following day. Appropriate corrections were made to account for the lipid and DNA background levels in the vasculature of the various organs.

Determination of Encapsulated DNA by the PicoGreen™ Fluorescence Assay

Plasmid encapsulation was evaluated by measuring the accessibility of the DNA-intercalating dye PicoGreen™ to plasmid at an excitation wavelength of 485nm and emission wavelength of 525nm (Aminco Bowman Series 2 Luminescence Spectrometer, SLM-Aminco, Urbana, IL). Typically 5 μl of PicoGreen was added to 1 ml of sample containing 0.2–1.0 μg plasmid. Plasmid encapsulation efficiency was calculated as $E(\%) = (I_0 - I)/I_0 \times 100$ where I and I_0 refer to the fluorescence intensities before and after the addition of Triton X-100 (final concentration 0.4%, v/v). Triton X-100 dissolves the SPLP exposing the encapsulated plasmid to the dye. Fluorescence intensities in the absence of PicoGreen were used as background references. The plasmid contents of formulations before and after DEAE column chromatography were determined by this assay. A standard curve was obtained using known quantities of the plasmid of interest in the presence and absence of Triton X-100 (Note, Triton did not affect the fluorescence intensity of the dye.) This assay was linear for up to 1 μg of double-stranded DNA.

Characterization and Quantification of Plasmid DNA in Tissue Extracts

Aliquots (100 μl) of homogenized tissue were added to 0.5 ml DNAzol in an Eppendorf tube, mixed gently by inverting, the tubes were topped up with 95% ethanol and incubated at 4°C overnight. Samples were centrifuged at ~10,000 $\times g$ for 15 min, the supernatant was discarded and 100 μl of TE was added. The sample was kept at 4°C for 2–3 days with occasional agitation to allow the DNA to dissolve. Ten microliter aliquots of dissolved DNA were analyzed by Southern and dot blot hybridization methods according to (Sambrook *et al.*, 1989b).

RESULTS

Formulations

Two SPLP formulations with different lipid composition were made. The major lipid constituents were POPC and cholesterol (PC/Chol-SPLP) or the fusogenic lipid DOPE (DOPE-SPLP). In both cases the particles were stabilized with 10% PEG-ceramide. Particles were characterized for their size and encapsulation efficiencies achieved. To obtain high plasmid entrapment with the different lipid compositions adjustments in the cationic lipid concentration were necessary. Optimal encapsulation was observed for PC/Chol SPLPs (40–50% plasmid encapsulation) with 12% DODAC (DODAC/POPC/Chol/PEG-CerC₂₀, 12/38.7/39.8/9.5 mol%) and for DOPE-SPLPs (60–70% encapsulation) with 6% DODAC (DODAC/DOPE/PEG-CerC₂₀, 6/84/10 mol%). The particle size in both formulations was approximately 100 nm with 75 ± 34 nm for DOPE-SPLPs and 118 ± 45 nm for PC/Chol-SPLPs as measured by dynamic light scattering. Further characterizations of SPLPs are outlined in detail elsewhere (Wheeler *et al.*, 1999).

Plasmid Protection in SPLPs

An important aspect of a delivery system designed for systemic application is its ability to retain its payload in an intact form during circulation. The integrity of the SPLPs was tested by determining the protection of the plasmid from degradation by serum nucleases. The different preparations (free plasmid, plasmid complexed with DOPE/DODAC LUVs, DOPE-SPLP and POPC/Chol-SPLP) were incubated in 90% mouse serum at 37°C for up to 5 h and the plasmid DNA characterized by Southern hybridization analysis. The percentage of intact DNA as a function of incubation time is shown in Fig. 1. Over 80% of the encapsulated plasmid in both SPLP preparations remained intact following a 5 h incubation period. However, free plasmid and plasmid complexed to DODAC/DOPE LUVs was degraded completely within 1 h.

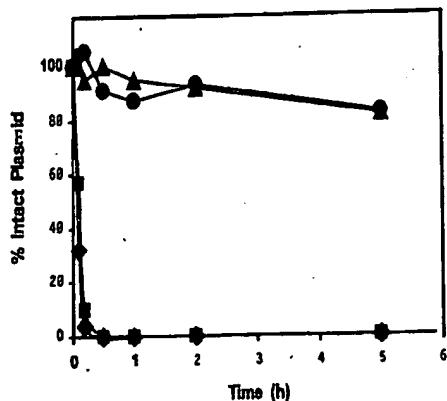


FIGURE 1 Protection of plasmid in DOPE-SPLP and POPC/Chol-SPLP from serum nucleases. Free plasmid (◊), plasmid complexed with DODAC/DOPE vesicles (■), DOPE-SPLP (●) and POPC/Chol-SPLP (▲) was incubated in 90% (v/v) normal mouse serum at 37°C for the time intervals as indicated. Following incubation the plasmid was extracted using DNazol and characterized by Southern hybridization analysis. Data are presented as percentage of intact DNA remaining (time 0 = 100%) and plotted as a function of incubation time.

Clearance and Biodistribution of SPLPs *In vivo* is Modulated by the PEG Anchor

Considerable circulation retention *in vivo* is necessary in order to achieve accumulation of SPLPs in a distal tumor (Gabizon and Papahadjopoulos, 1988). Previous studies have shown that circulation longevity can be achieved with liposomes coated with PEG (Parr *et al.*, 1997). However, the presence of the PEG coating is expected to inhibit the association and fusion of the SPLPs with cells in a similar manner to the inhibition of fusion between LUVs by PEG coatings (Holland *et al.*, 1996). In that study it was shown that LUVs containing PEG linked to a lipid anchor with short acyl chains that permits exchange of PEG out of the LUVs, will become increasingly fusogenic with the loss of PEG. Here, we studied the effect of PEG anchored to ceramide of different acyl chain lengths (CerC₁₄ and CerC₂₀) on the clearance of the SPLP. As shown in Fig. 2(a) POPC/Chol-SPLPs containing PEG-CerC₂₀ exhibit an extended circulation lifetime

($t_{1/2} \sim 10$ h) whereas SPLPs with PEG-CerC₁₄ are cleared rapidly ($t_{1/2} \sim 1$ h). The clearance profile observed for PE-SPLPs with PEG-CerC₁₄ (data not shown) and PEG-CerC₂₀ (Fig. 4(a)) were similar to the corresponding PC/Chol-SPLPs.

The different circulation longevity of the SPLPs is reflected in the lipid accumulation in the organs as summarized in Table I. Particles with the shorter hydrophobic anchor PEG-CerC₁₄ are cleared mainly by the liver which accounts for $\sim 50\%$ of the injected dose at the 1 h time point. Accumulation in the spleen at this time is approximately 2% of injected dose. Only minute levels of the lipid marker

were detected in lung, heart and kidney. For particles with PEG-CerC₂₀ approximately 20% and 1% of the injected lipid dose appeared in the liver and spleen respectively, at the 1 h time point. Again negligible amounts of the labeled lipids were detected in other organs.

Integrity of Plasmid in SPLP in the Circulation

The clearance properties of the SPLPs based on a lipid marker alone do not provide information about the stability of the particle and its payload. To evaluate the integrity of the plasmid inside the

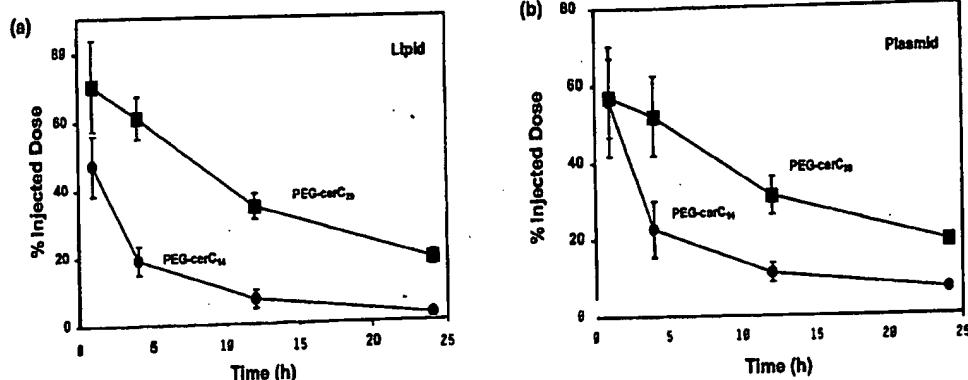


FIGURE 2 Clearance of POPC/Chol-SPLP stabilized with PEG-CerC₁₄ and PEG-CerC₂₀ from blood following iv injection. POPC/Chol-SPLP with PEG-CerC₁₄ (○) and POPC/Chol-SPLP with PEG-CerC₂₀ (■) were injected into CD-1 mice at a dose of 30 µg plasmid and ~ 1 mg 3 H-lipid. Mice were sacrificed 1, 4, 12 and 24 h following injection. Plasma samples were analyzed for radioactivity (3 H-lipid) by scintillation counting and for intact plasmid by Southern hybridization analysis as described in Methods. Data are expressed as percent injected dose of lipids (a) and plasmid (b) remaining in the plasma and plotted as a function of time. The plasmid dose was estimated based on encapsulation efficiency.

TABLE I Biodistribution of PC/Chol-SPLP in CD-1 mice

Lipid-DNA particle	Time post-injection (h)	% Injected dose					
		Plasma (SD)	Spleen (SD)	Liver (SD)	Lung (SD)	Heart (SD)	Kidney (SD)
SPLP-CerC ₂₀	1	70.5 (13.2)	0.96 (0.78)	21.23 (8.21)	0.29 (0.34)	0.58 (0.19)	-0.06 (0.08)
	4	61.02 (6.32)	0.74 (0.04)	18.78 (4.01)	0.38 (0.39)	0.86 (0.20)	0.46 (0.16)
	12	34.39 (3.88)	0.96 (0.34)	18.83 (1.38)	0.32 (0.04)	0.39 (0.06)	0.77 (0.26)
	24	18.46 (2.14)	1.5 (0.19)	23.37 (6.16)	0.21 (0.26)	0.25 (0.12)	1.31 (0.14)
SPLP-CerC ₁₄	1	47.18 (8.84)	1.22 (0.8)	48.03 (2.13)	1.13 (0.48)	0.44 (0.28)	0.36 (0.25)
	4	18.93 (4.19)	2.18 (0.77)	56.87 (4.7)	0.55 (0.10)	0.18 (0.04)	0.50 (0.22)
	12	7.20 (2.68)	2.07 (0.39)	48.61 (12.59)	0.37 (0.07)	0.18 (0.06)	0.66 (0.20)
	24	2.17 (0.84)	1.99 (0.46)	55.39 (7.59)	0.39 (0.10)	0.14 (0.05)	0.66 (0.05)

particles during circulation, plasmid DNA recovered from blood samples at various times following injection were analyzed by Southern blot hybridization methods. The amount of intact plasmid decreases over time and as shown in Fig. 2(b), the plasmid clearance profile reflects the lipid clearance from the blood. Intact plasmid was detected in the plasma of mice injected with PC/Chol-SPLPs with PEG-CerC₁₄ and PEG-CerC₂₀ even 24 h after administration (see Fig. 3). This clearly indicates that plasmid is retained in the SPLPs and is fully protected from degradation by serum nucleases.

The DOPE-SPLPs exhibit similar stability and plasmid protection as PC/Chol-SPLP (Fig. 4). In this case, radiolabeled lipid (¹⁴C-CHE) and DNA (³H-Plasmid) were used to follow the fate of the

respective components. The DNA to lipid ratio in the samples remained constant (~ 1) at all time points (Fig. 4(b)) suggesting stable particles and retention of plasmid in the circulation. Southern hybridization analysis confirmed the integrity of the DNA (see below for BDF-1 mice, Fig. 6).

DOPE-SPLP Biodeistribution and Delivery of Intact Plasmid to Organs

The tissue accumulation of DNA and lipid from DOPE-SPLPs with PEG-CerC₂₀ is summarized in Table II. We find that both lipid and plasmid levels in the spleen are consistently low. The amount of lipid detected in the liver increases slowly over the 24 h period (24% of injected dose after 24 h),

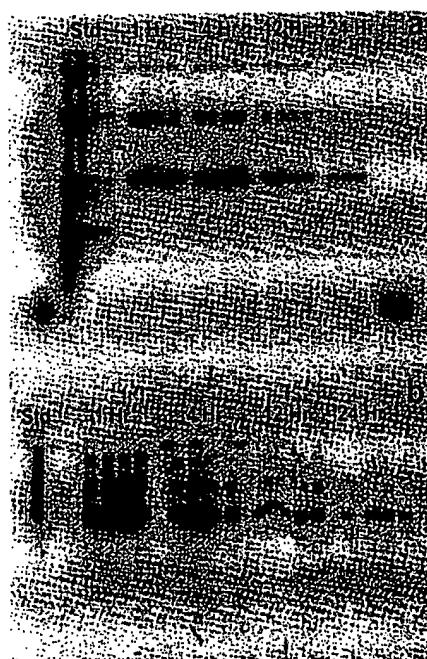


FIGURE 3 Integrity of plasmid in the SPLP recovered from the blood after iv injection of SPLP. Southern hybridization analysis of plasmid DNA recovered from the blood of CD-1 mice 1, 4, 12 and 24 h following injection of (a) POPC/Chol-SPLP with PEG-CerC₂₀ and (b) POPC/Chol-SPLP with PEG-CerC₁₄. The plasmid was recovered from plasma samples using DMAzol and assayed by Southern hybridization.

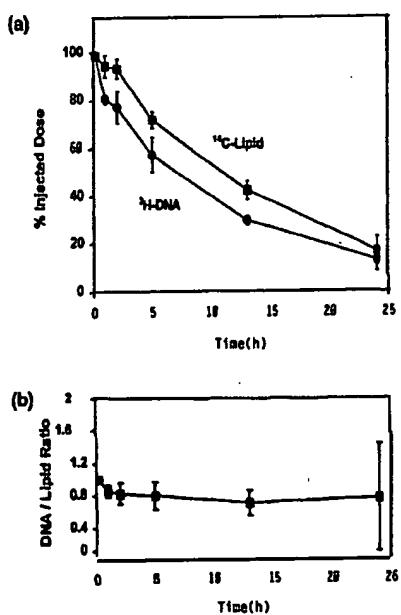


FIGURE 4 Clearance of DOPE-SPLP from blood following iv injection. DOPE-SPLP (2 mg ¹⁴C-lipid; 30 μ g ³H-DNA) was injected into CD-1 mice. Mice were sacrificed 5 min, 1, 2, 5, 12 and 24 h after injection. Plasma samples were analyzed for radioactivity (³H-DNA and ¹⁴C-lipids) by scintillation counting. Data are expressed as percent injected dose of lipids (■) and plasmid (●) remaining in the plasma and plotted as a function of time in (a). The DNA to lipid ratio (% dose/% dose) as a function of time is plotted in (b).

TABLE II Biodistribution of [³H]-DNA encapsulated in [¹⁴C]-DOPE-SPLP (CerC₂₀) in CD-I mice

Time	Marker	% Injected dose					
		Blood	Spleen	Liver	Lung	Heart	Total
5 min	[³ H]	98.7 (1.2)	0.0 (0.0)	1.5 (0.0)	0.1 (0.3)	1.9 (0.1)	0.0 (0.0)
	[¹⁴ C]	98.7 (1.0)	0.0 (0.0)	0.1 (0.4)	0.3 (0.1)	1.9 (0.0)	0.0 (0.0)
	[³ H]/[¹⁴ C]	1.000 (0.003)					102.0 (0.5)
1 h	[³ H]	80.6 (2.0)	0.4 (0.6)	2.6 (0.4)	0.6 (0.1)	1.2 (0.1)	0.2 (0.5)
	[¹⁴ C]	94.4 (4.7)	0.0 (0.2)	3.7 (0.1)	0.5 (0.1)	1.2 (0.1)	0.0 (0.2)
	[³ H]/[¹⁴ C]	0.855 (0.028)					85.7 (2.1)
2 h	[³ H]	17.3 (6.7)	1.2 (0.9)	2.9 (1.4)	0.5 (0.3)	1.2 (0.3)	0.2 (0.3)
	[¹⁴ C]	93.4 (4.1)	0.0 (0.2)	5.9 (1.1)	0.7 (0.2)	1.1 (0.3)	0.0 (0.0)
	[³ H]/[¹⁴ C]	0.827 (0.039)					83.3 (6.7)
5 h	[³ H]	57.4 (7.2)	1.3 (0.2)	2.6 (1.3)	0.5 (0.2)	0.8 (0.3)	0.7 (0.3)
	[¹⁴ C]	71.9 (3.5)	0.3 (0.2)	9.0 (2.0)	0.8 (0.3)	0.8 (0.3)	0.3 (0.3)
	[³ H]/[¹⁴ C]	0.797 (0.078)					83.2 (3.4)
13 h	[³ H]	30.0 (1.9)	1.3 (0.2)	3.0 (0.5)	0.4 (0.1)	0.6 (0.3)	1.2 (0.5)
	[¹⁴ C]	42.6 (4.0)	1.3 (0.1)	18.0 (2.2)	0.7 (0.1)	0.6 (0.2)	1.6 (0.4)
	[³ H]/[¹⁴ C]	0.706 (0.023)					36.5 (2.0)
24 h	[³ H]	13.2 (4.4)	1.1 (0.2)	3.5 (0.2)	0.5 (0.1)	0.5 (0.0)	1.4 (0.2)
	[¹⁴ C]	17.1 (5.7)	2.1 (0.2)	23.5 (2.4)	0.6 (0.1)	0.5 (0.1)	3.1 (0.7)
	[³ H]/[¹⁴ C]	0.768 (0.004)					20.2 (4.6)

however, the amount of plasmid detected at all time points remains at approximately 3-4% of the injected dose. This probably reflects the relative rates of degradation of the ³H-plasmid and ¹⁴C-CHE in the liver over time. To investigate this possibility further we characterized the plasmid DNA extracted from the liver and spleen. For this study SPLPs with PEG-CerC₁₄ were chosen since they showed the highest accumulation in the liver and spleen. Tissues derived from animals treated with PC/Chol-SPLPs containing PEG-CerC₁₄ were examined by Southern hybridization analysis (Fig. 5(a)). Clearly, significant quantities of intact plasmid were detected in the liver and spleen at all time points. In the liver, plasmid DNA appears to be degraded very quickly over time while in the spleen the degradation occurs at a much slower rate (Fig. 5(b)). This is consistent with the results from the SPLPs labeled with radioisotopes only shown above. A similar plasmid distribution was also observed for DOPE-SPLPs with PEG-CerC₁₄, thus the Southern Blot and the DNA profile shown in Fig. 5 are representative of the fate of plasmid DNA delivered by the SPLPs.

Pharmacokinetics of SPLPs in Normal and Tumor-Bearing BDF-1 Mice

The murine fibrosarcoma line and mouse model were chosen largely for the high degree of vascularization of the tumor *in vivo* and since tumor propagation in BDF-1 mice has been shown previously to be highly successful (Harasym *et al.*, 1997). SPLPs with PEG-CerC₂₀ were used for these studies as they exhibited the most extended circulation lifetimes (Figs. 2 and 4). The pharmacokinetics for DOPE-SPLP and PC/Chol-SPLP with PEG-CerC₂₀ were determined in non-tumor bearing and tumor-bearing BDF-1 mice and compared to that of free plasmid and plasmid complexed to DODAC/DOPE LUVs.

In normal BDF-1 mice the SPLP are cleared from the circulation at a similar rate as in CD-I mice. As an example, the clearance profile for DOPE-SPLPs is shown in Fig. 6. Blood samples were analyzed for intact plasmid at different time intervals following injection by Southern hybridization analysis. As observed previously in CD-I mice (Fig. 4) plasmid and lipid were removed from the blood

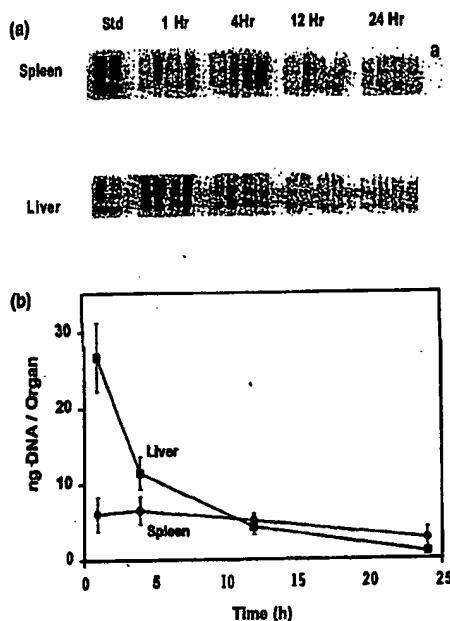


FIGURE 5 Southern hybridization analysis of plasmid DNA extracted from spleen and liver. CD-1 mice were injected iv with POPC/Chol-SPLP with PEG-CerC₁₄ and sacrificed 1, 4, 12 and 24 h post-injection. Plasmid was extracted from spleen and liver homogenates and analyzed by Southern hybridization (a). In (b) the amount of intact plasmid recovered in liver (■) and spleen (●) is plotted for different time points following injection.

concomitantly. The biodistribution for DOPE-SPLPs and PC/Chol-SPLPs are summarized in Table III for the 1 and 24 h time points post-injection and compared to DODAC/DOPE-plasmid complexes and free plasmid. Accumulation in the liver and spleen are listed separately while for the lung, heart and kidney it is combined together as "others" since the SPLP accumulation in these organs was consistently minimal. As was expected, the DODAC/DOPE-plasmid complexes were cleared almost completely from the blood, primarily by the liver, within 1 h following injection. The two encapsulated formulations, however, maintained their integrity in the blood with approximately 60% and 86% of PC/Chol-SPLP and DOPE-SPLP, respectively, remaining after 1 h and between 10% and 20% after 24 h with approximately an equivalent amount of intact plasmid.

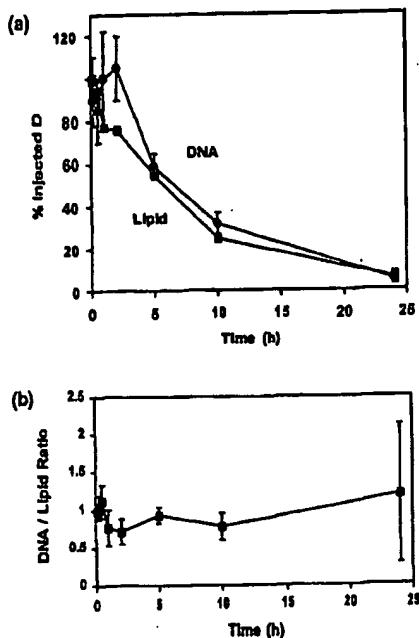


FIGURE 6 Plasma clearance of DOPE-SPLP in BDF-1 mice. DOPE-SPLP was injected into normal BDF-1 mice at 30 µg plasmid DNA per mouse. Mice were sacrificed at 0.1, 0.2, 0.5, 1, 2, 5, 10, 24 h after injection. Plasma samples were analyzed for ³H-labeled lipid by scintillation counting. Plasmid DNA was recovered from the plasma using DNazol and assayed by Southern hybridization analysis. Data are expressed as percent of injected dose of lipid (■) and intact plasmid (●) remaining in the plasma and plotted as a function of time after injection (a). Bars represent SD (*n* = 3). The DNA to lipid ratio (% dose/% dose) as a function of time is plotted in (b).

Delivery of Intact Plasmid to a Subcutaneous Lewis Lung Carcinoma

The pharmacokinetics of the DOPE-SPLPs in BDF-1 mice bearing a Lewis lung carcinoma in the left hind flank (Fig. 7(a)) appears to be similar to that in non-tumor bearing animals. Accumulation of remarkably high levels of lipid (10% of injected dose) and intact plasmid (6% of injected dose) were observed in the tumor over the first 10 h following bolus injection into the lateral tail vein (Fig. 7(b)). Approximately 4% of the plasmid dose was still detected 24 h following injection.

The accumulation of lipid and plasmid of DOPE-SPLPs in tumor was compared to the accumulation obtained with PC/Chol-SPLPs and

TABLE III Biodistribution of different liposomal formulations with plasmid DNA in normal BDF-1 mice^a

Plasmid formulation ^b	Time (h)	% Injected dose ^c				
		Plasmid DNA		³ H-Lipid		
		Plasma	Plasma	Liver	Spleen	Others ^d
(A) Free plasmid DNA	1	2.4 (1.3)	n/a	n/a	n/a	n/a
	24	1.5 (0.4)	n/a	n/a	n/a	n/a
(B) DOPE/DODAC-LUVs	1	2.3 (0.9)	32 (1.3)	43.5 (0.4)	4.1 (0.6)	3.7 (0.4)
	24	2.8 (0.2)	3.3 (2.5)	45.8 (3.5)	4.1 (1.0)	2.4 (0.3)
(C) DOPE/DODAC/PEG-Cer (C ₂₀) SPLP	1	82.9 (6.9)	85.9 (2.1)	2.7 (0.3)	0.3 (0.0)	1.7 (0.2)
	24	15.7 (0.3)	11.0 (3.3)	23.0 (2.3)	2.1 (0.1)	1.6 (0.1)
(D) POPC/Chol/DODAC/PEG-Cer (C ₂₀) SPLP	1	69.3 (27.2)	59.5 (6.0)	21.0 (1.5)	1.9 (0.3)	1.6 (0.4)
	24	14.5 (2.2)	23.2 (6.0)	29.4 (5.4)	2.2 (0.8)	1.6 (0.4)

^aDose: 30 µg plasmid DNA and ~1–2 mg lipid per mouse. ^bPINEXCAT plasmid DNA was either complexed with LUVs (B) or encapsulated in SPLP (C) and (D). ^cData are expressed as the percentage of injected dose of lipid or plasmid DNA together with SD (*n* = 3). ^dDNA was determined by Southern blot analysis and lipid by the radiolabel ³H-CHE. ^dOthers include lungs, heart and kidneys.

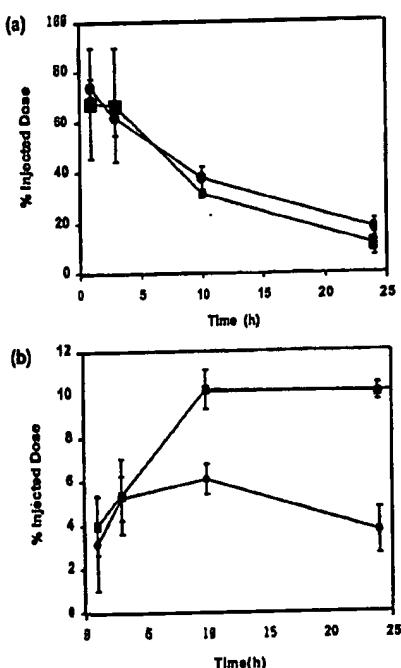


FIGURE 7 Tumor accumulation of and plasma clearance of DOPE-SPLP in BDF-1 mice bearing Lewis lung tumor. Mice were seeded with tumor cells and after 14 days injected with DOPE-SPLP at a dose of 30 µg plasmid DNA and ~2 mg lipid. Animals were sacrificed 1, 3, 10 and 24 h post-injection. Plasma samples (a) and tumor tissue (b) were analyzed for ³H-lipids by scintillation counting and for intact plasmid by Southern hybridization analysis. The amount of lipid (■) and intact plasmid (●) recovered from tumor tissue and blood are given as percent injected dose and plotted as a function of time following injection.

DODAC/DOPE-plasmid complexes (Table IV). The amount of intact plasmid detected in the tumor for PC/Chol-SPLPs (4.4% ± 0.7%) 10 h post-injection was not significantly different to that for DOPE-SPLPs (6.1% ± 0.7%). Only trace amounts of plasmid (< 0.1%) were detected in the tumor following injection of DOPE/DODAC-plasmid complexes. The percentage of SPLPs remaining in the circulation 10 h post-injection was lower for PC/Chol-SPLPs. However, this is likely due to differences in the lipid dose injected. The lipid dose was lower for the PC/Chol-SPLPs than for the DOPE-SPLPs and probably resulted in a somewhat faster clearance, similar to the liposomal clearance rates previously reported for different lipid doses (Oja *et al.* 1996). The apparent accumulation of plasmid and lipid diverge over time and could reflect the processing of the SPLP in tumor tissue indicating a faster metabolism of the plasmid as compared to the lipid.

DISCUSSION

The primary objective of this study was to develop a gene delivery system for systemic application that results in preferential accumulation at disease sites such as inflammation or tumor. It has been demonstrated previously that the delivery of anti-cancer

TABLE IV Tumor and plasma levels of different liposomal formulations of plasmid DNA following iv injection^a

Plasmid formulation ^b	% Injected dose ^c			
	Tumor		Plasma	
	Plasmid DNA	Lipid	Plasmid DNA	Lipid
(A) Free plasmid DNA	0.1 (0.3)	n/a	0.0 (0.4)	n/a
(B) DOPE/DODAC LUVs	0.1 (0.4)	1.1 (0.2)	0.0 (0.3)	2.5 (0.3)
(C) DOPE/DODAC/PEG-Cer (C20) SPLP	6.1 (0.7)	10.2 (0.9)	37.9 (4.5)	31.5 (1.1)
(D) POPC/Chol/DODAC/PEG-Cer (C20) SPLP	4.4 (0.7)	5.3 (0.7)	9.9 (3.7)	14.6 (1.5)

^a50 µg plasmid DNA was injected per mouse as free plasmid or liposomal formulations as indicated. Mice were sacrificed 10 h following injection. Tumor tissues and plasma samples were analyzed for plasmid DNA by Southern hybridization analysis and for lipid by scintillation counting.

^bpINEXCAT plasmid DNA was either complexed with LUVs (B) or encapsulated in SPLP (C) and (D).

^cData are expressed as the percentage of injected dose of liposomes or plasmid DNA together with SD (*n* = 3). Data for tumor tissues are presented as percentages of injected dose per gram tissue.

drugs to tumor tissue can be achieved with carrier systems where the drug of interest is encapsulated in 100 nm diameter liposomes (LUVs). These vesicles evade uptake by the RES resulting in extended circulation lifetimes and have been shown to accumulate at tumor sites (Gabizon and Papahadjopoulos, 1988; 1992; Allen and Hansen, 1991). The increased accumulation was attributed to enhanced permeability of the tumor vasculature allowing extravasation of small particles into the surrounding tissue (Wu *et al.*, 1993; Hobbs *et al.*, 1998; Monsky *et al.*, 1999). Based on these observations, the following requirements are expected for a lipid based plasmid delivery system targeting these disease sites: (I) extended circulation lifetime, (II) small size of approximately 100 nm, (III) highly stable particles providing full protection of the plasmid from degradation by serum nucleases, (IV) particles able to interact with and enter into target cells upon arrival at the disease site and (V) efficient intracellular delivery of plasmid.

Presently, the favored delivery systems for gene transfer are viral systems since they best address the latter two requirements. The engineered viruses are efficient at transferring foreign genes into cells, however, they are highly immunogenic and consequently of limited use particularly for repeat systemic application. The most widely used non-viral gene delivery systems are DNA-lipid complexes formed by mixing plasmid DNA with lipid vesicles (SUVs or LUVs) composed of a cationic lipid

derivative and DOPE as a helper lipid. The complexes formed are capable of transporting the plasmid across cell membranes achieving delivery into a wide spectrum of cells *in vitro*, a process known as lipofection. The major drawbacks of these systems are their tendency to aggregate in serum, the short circulation lifetime and the limited protection of the plasmid from degradation by serum nucleases (Fig. 1) (Lew *et al.*, 1995; Mahato *et al.*, 1995). The fast clearance of the DNA-lipid complexes from the blood is not surprising since charged particles are known to bind serum proteins targeting them for removal by the phagocytic cells of the RES (Allen *et al.*, 1984; Illum and Davis, 1983; Chonn *et al.*, 1991; 1992; Semple *et al.*, 1998). Furthermore, large complexes and aggregates can be trapped in the lung capillaries. The latter will result in an extended exposure of the lung endothelial bed to DNA-lipid complexes and is most likely the reason for the preferential transfection observed in the lung following iv administration of these complexes (Templer *et al.*, 1997; Liu *et al.*, 1997).

This paper addresses the first three requirements; of a plasmid delivery system to access disease sites as discussed above. The strategy was to encapsulate, the DNA inside a lipid shell forming DNA-lipid particles of ~100 nm diameter in order to achieve extended circulation lifetimes as well as protection of the plasmid DNA. The PEG-lipid derivatives included in our SPLP play a key role in attaining these desired features. Initially the PEG lipid

stabilizes the particles containing a high concentration of the fusogenic lipid DOPE. Following injection into the blood stream PEG shields the positively charged lipid DODAC, required for efficient encapsulation, from interactions with serum proteins known to cause rapid clearance of lipid vesicles (Semple *et al.*, 1998). LUVs with GM1 (Allen and Chonn, 1987; Gabizon and Papahadjopoulos, 1988) or PEG-PE (Allen *et al.*, 1991; Parr *et al.*, 1994) amphiphiles included in the membrane were shown to exhibit extended circulation times by successfully avoiding uptake by the RES. A potential drawback of the polymer coating of the particles is a reduced particle-cell surface interaction, which is necessary to induce cellular uptake. Therefore, a polymer coating was designed that dissociates from the particles over time. It was expected that these polymer-coated carriers could initially evade uptake by the RES resulting in prolonged circulation lifetime and leading to accumulation at disease sites. Following exchange of the polymer from the carrier, cellular uptake can then occur. Inclusion of PEG-PE in these SPLPs was not considered, primarily because its negative charge interferes with DNA encapsulation and also based on previous results, which indicate that the exchange rate of PEG-PE from liposomes is too slow (Mori *et al.*, 1998) presumably due to electrostatic attraction to the cationic lipid DODAC. Employing a neutral PEG-lipid circumvented these problems. Ceramide was chosen as the lipid anchor since the anchor strength can be varied by using a ceramide backbone with different acyl chain lengths. An increase in the acyl chain length of the ceramide will increase the hydrophobicity of the anchor resulting in a lower exchange rate of PEG as shown in an *in vitro* model system (Holland *et al.*, 2000). Previously we have shown that the transfection potential of the SPLP *in vitro* is indeed dependent on the depletion of the PEG-coating. SPLP with PEG-CerC₂₀, which has a long residence time in the lipid bilayer, showed low transfection whereas greatly improved transfection was obtained with SPLP containing PEG-CerC₁₄ that dissociates much more rapidly (Wheeler *et al.*, 1999).

In this study two ceramide anchors were evaluated (CerC₁₄ and CerC₂₀). The clearance of the SPLP is evidently related to the PEG anchor used as shown in Fig. 2. Circulation longevity was achieved with SPLPs containing PEG-CerC₂₀ while SPLPs with PEG-CerC₁₄ are cleared from the blood much more rapidly. This is consistent with the accelerated exchange rate observed for the shorter PEG ceramide anchor C₁₄ compared to that of C₂₀ with $t_{1/2}$ of 1.2 and several days, respectively (Wheeler *et al.*, 1999) and demonstrates the possibility of modulating the circulation lifetime of the SPLP. The SPLPs are stable in the blood and protect the plasmid during circulation as indicated by the observed plasmid-to-lipid ratio of approximately 1 detected in blood samples recovered after different time intervals following injection for SPLP with PEG-CerC₁₄ and PEG-CerC₂₀, respectively (Figs. 4(b) and 6(b)). The virtually complete protection of the plasmid in the SPLP during circulation in the blood is a key feature of these particles and was not obtained with any of the previously described lipid carriers.

The SPLPs are cleared from the blood mainly by the liver since the accumulation in this organ of 50% and 20% 1 h after injection for SPLP with PEG-CerC₁₄ and PEG-CerC₂₀, respectively, mirrors the clearance of these particles (Table I). The liver accumulation values for both the PC/Chol and DOPE-based SPLPs with the PEG-CerC₂₀ derivative in CD-1, BDF-1 and BDF-1-tumor-bearing mice are very similar to those attained previously with liposomes containing PEG-PE (Allen *et al.*, 1991; Parr *et al.*, 1994) or GM1 (Allen and Chonn, 1987; Gabizon and Papahadjopoulos, 1988; 1992). Note that there was no significant accumulation of any of the SPLPs in the lung as predicted for particles of this size. Importantly, intact plasmid was delivered by the SPLP to liver and spleen and the slow degradation observed over time is suggestive of the bioavailability of the plasmid at the site.

Finally, the SPLPs with extended circulation lifetimes show significant accumulation in Lewis Lung Carcinoma tissue following iv administration and delivery of intact plasmid (6% of injected dose).

The lipid accumulation detected in tumor tissue (approximately 10% of injected dose) is similar to the highest levels previously reported (6-10%) for liposomes that exhibit comparable pharmacokinetics to the SPLP (Allen and Chonn, 1987; Gabizon and Papahadjopoulos, 1988; 1992; Allen *et al.*, 1991; Parr *et al.*, 1994). These data confirm that circulation longevity is indeed an important parameter for a carrier system to deliver plasmid successfully to tumor tissue. The accumulation of intact plasmid in the tumor mass is clearly substantial, but the level of gene expression observed in tumor tissue was low (data not shown). However, the long residence times (10-24 h) of the SPLPs in tumor tissue following the bolus injection indicates that the SPLPs did extravasate into the interstitial space of the tumor and are available for uptake by tumor cells. The accelerated decrease of intact plasmid in the tumor tissue as compared to the lipid indicates processing of the SPLPs in the tumor tissue and bioavailability of the plasmid. The released plasmid is broken down faster by nucleases than the lipid marker is metabolized. Most likely, the SPLPs are taken up preferentially by phagocytic host-cells and macrophages. Incorporation of ligands into the SPLP for specific and increased uptake by tumor cells might be required.

In summary, the SPLP described here fulfill the first three requirements for a plasmid delivery system outlined earlier. We have demonstrated delivery of intact plasmid to a distal tumor site with a lipid based carrier system following systemic administration. The plasmid is encapsulated inside a lipid bilayer and therefore fully protected from degradation. The dissociable polymer coating of the SPLPs allows the particles to avoid the clearance by the RES leading to accumulation in tumor tissue. The delivery of intact plasmid into tumor tissue represents the first step in the development of an efficient gene carrier system with application in cancer gene therapy. Presently, the cellular uptake of the carrier at the disease site, identification of cell types and the fate of the plasmid are evaluated as critical steps towards the final goal, the efficient expression of a desired gene.

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